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**Synthesis of cyclic peptide natural products and peptidomimetics**

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# Synthesis of Cyclic Peptide Natural Products and Peptidomimetics

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A thesis submitted for the degree of Doctor of Philosophy

University of Bath

Department of Pharmacy and Pharmacology

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These research studies were carried out under the supervision of Dr. Ian Eggleston.



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## Abstract

Chitin, a linear polymer of *N*-acetylglucosamine, is an essential structural component of fungal, nematode and insect pathogens but is not found in human physiology. Chitinases, which hydrolyze this polymer, play a key role in life cycle of these pathogens and associated pathogenesis. Consequently chitinase inhibitors have generated a lot of interest given their potential as insecticides, fungicides and antimalarials.

Herein, approaches are reported to the synthesis of some non-sugar based chitinase inhibitors: the cyclic pentapeptide natural products argifin, banyasin A and diketopiperazines related to the natural product CI-4.

In order to improve the efficiency of production of argifin and facilitate SAR on analogues of the natural product, a revised synthesis of argifin has been developed.

The synthesis of argifin was carried out by a combination of solid-phase and solution chemistry. The assembly of the linear peptide was carried out by SPPS and the cyclisation was performed in solution. The protecting groups chosen for the Asp and Arg residues were removable by hydrogenolysis, as this allowed aspartimide formation under acidic conditions to be avoided. Only one HPLC purification was required at the final step; argifin was isolated in 19% yield, compared to 18% yield for the first synthesis by Dixon *et al.*

Banyasin A contains the same essential Arg(MC)-MePhe dipeptide motif as argifin and so it is of considerable interest as a potential Family 18 chitinase inhibitor. The synthesis of Amoa (3-amino-2-methyl-5*E*-octenoic acid) a rare amino-acid present in banyasin A was investigated. An advanced intermediate for the synthesis of Amoa was successfully obtained *via* chiral pool chemistry in an 8 step sequence from L-Asp. This involved preparation of a selectively protected  $\beta$ -methyl-substituted Asp derivative, which was then homologated to the  $\beta$ -amino-acid *via* Arndt-Eistert chemistry to give (3*R*,4*R*)-3-(((benzyloxy)carbonyl)amino)-4-methyl-5-oxo-5-allyloxypentanoic acid in 27% yield for the final step.

The cyclic dipeptide CI-4, cyclo (L-Arg-D-Pro) is a weak inhibitor ( $IC_{50} = 1.2$  mM) of Family 18 chitinases, however its binding efficiency index (BEI) is comparable to more potent inhibitors such as argifin. Some analogues of CI-4 show promising activity against a typical bacterial type Family 18 chitinase, *SmChiB1* from *Serratia marcescens*. The cyclic dipeptide should therefore be a useful starting point for the development of more effective and selective inhibitors of this enzyme class. A series of cyclo (Xaa-Pro)-based dipeptides were synthesized,

with different Xaa such as L/D-Pro, Gly, L-Ser, L/D-Arg, D-His, D-Phe, with yields ranging from 12 to 84%. Preliminary biological data confirm that cyclo (D-Xaa-D-Pro) may be a novel template for the development of new drug-like inhibitors of Family 18 chitinases.

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## List of abbreviations

Ac	acetyl
AcOH	acetic acid
ALLO	allosamidin
Aq	aqueous
Ar	aryl
Arg	arginine
Asp	aspartic acid
BEI	binding efficiency indices
Boc	<i>tert</i> -butoxycarbonyl
BnOCOC1	benzyl chloroformate
CSA	10-camphorsulfonic acid
Da	Dalton
DBU	1,8-diazabicycloundec-7-ene
DCC	<i>N,N'</i> -dicyclohexylcarbodiimide
DCM	dichloromethane
Dde	1-(4,4-dimethyl-2,6-dioxohexylidene)ethyl
DEAD	diethyl azodicarboxylate
DEME	demethylallosamidin
DIBAL-H	diisobutylaluminium hydride
DIC	<i>N,N'</i> -diisopropylcarbodiimide
DIPEA	<i>N,N</i> -diisopropylethylamine
DKP	diketopiperazine
DMAP	4-(dimethylaminopyridine)
DMF	<i>N,N</i> -dimethylformamide
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DPPA	diphenylphosphoryl azide
EDC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
Eq	equivalent(s)
EMS	electrospray mass spectrometry
Et <sub>2</sub> O	diethyl ether
EtOAc	ethyl acetate

EtOH	ethanol
ETP	epithiodioxopiperazine
ESI	electrospray ionisation
FBS	fragment-based screening
Fmoc	9-fluorenylmethoxycarbonyl
Fig	figure
g	grams
Glu	glutamic acid
GLCB	glucoallosamidin
Gly	glycine
h	hour
HATU	(1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxide hexafluorophosphate
His	histidine
HTS	high-throughput screening
HOBt	1-hydroxybenzotriazole
HPLC	high performance liquid chromatography
Hz	Hertz
IC <sub>50</sub>	concentration required for 50% inhibition of activity
IP	isoelectric point
IPCC	isopropenyl chlorocarbonate
IR	infra-red
<i>J</i>	coupling constant
<i>K<sub>i</sub></i>	inhibition constant
LE	ligand efficiency
LLE	ligand-lipophilic efficiency
Lit	literature
M	molar
METH	methylallosamidin
Mp	melting point
<i>m/z</i>	mass to charge ratio (mass spectrometry)
MC	methylcarbamoyl
Me	methyl
MeOH	methanol

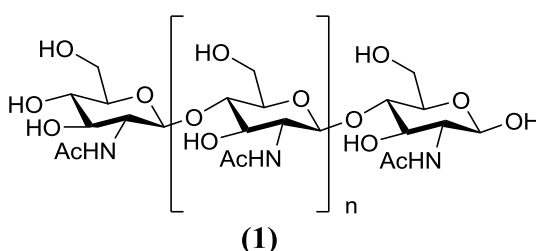
MePhe	<i>N</i> -methylphenylalanine
Min	minutes
mL	millilitres
NMM	<i>N</i> -methylmorpholine
mmol	millimoles
mol	moles
MU	4-methylumbelliferone
mRNA	messenger ribonucleic acid
MsCl	methanesulfonyl chloride
NAG	<i>N</i> -acetylglucosamine
NEM	<i>N</i> -ethylmorpholine
<i>n</i> -BuLi	<i>n</i> -butyl lithium
nM	nanomolar
NMP	<i>N</i> -methyl-2-pyrrolidinone
NMR	nuclear magnetic resonance
OAlI	<i>O</i> -allyl
OBn	<i>O</i> -benzyl
Orn	ornithine
PCR	polymerase chain reaction
PPA	propane phosphonic acid anhydride
PEST	proline (P), glutamic acid (E), serine (S), threonine (T)-rich sequence
Petrol	petroleum ether 40-60 °C
Ph	phenyl
Phe	phenylalanine
PhFl	9-phenylfluoren-9-yl
PMB	<i>para</i> -methoxybenzyl
Pmc	2,2,5,7,8-pentamethylchroman-6-sulfonyl
PMN	polymorphonuclear neutrophils
ppm	parts per million
PR	pathogenesis-related proteins
Pro	proline
<i>p</i> -TsCl	<i>para</i> -toluenesulfonyl chloride
PyBOP	(benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate
PyBrOP	bromotripyrrolidinophosphonium hexafluorophosphate

QS	quorum sensing
R <sub>f</sub>	retention factor
R <sub>t</sub>	retention time
RNA	ribonucleic acid
RP-HPLC	reversed-phase high-performance chromatography
ROS	reactive oxygen species
SAR	structure activity relationship
Ser	serine
SPPS	solid-phase peptide synthesis
TBAI	tetrabutylammonium iodide
TBTU	2-(1H-Benzotriazol-1-yl)-1,1,3,3-tetramethylaminium tetrafluoroborate
Tert	tertiary
TFA	trifluoroacetic acid
TFAA	trifluoroacetic acid anhydride
THF	tetrahydrofuran
THP	tetrahydropyranyl
TIM	triosephosphate isomerase
TIPS	triisopropylsilyl
TLC	thin layer chromatography
TMEDA	tetramethylethylenediamine
TMSCHN <sub>2</sub>	trimethylsilyldiazomethane
TMSE	2-(trimethylsilyl)ethyl
Trp	tryptophan
Tyr	tyrosine
UV	ultraviolet
VS	virtual screening
Xaa	amino-acid
Z	benzyloxycarbonyl

# 1. Introduction

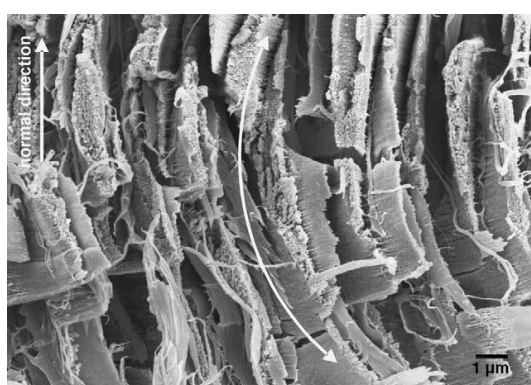
## 1.1. Chitin

Chitin is a linear polymer of  $\beta$ -1,4-linked *N*-acetyl-glucosamine (Figure 1),<sup>1</sup> which is, after cellulose, the most abundant polysaccharide in nature. It is present as a structural component in the coatings of many species, such as the cell wall of most fungi<sup>2</sup>, the microfilarial sheath of parasitic nematodes<sup>3</sup>, sheaths of crustaceans and exoskeletons of all types of arthropods.<sup>4</sup> Chitin is also a part of the the cuticle of the epidermis and trachea, stomodeum and proctodeum of many insects.<sup>5</sup>

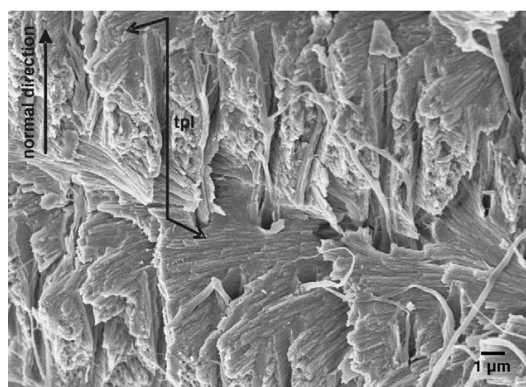


**Figure 1:** Structure of chitin

Chitin is a significant component of the integument or exoskeleton of insects. Various analyses show that the chitin content of the cuticle, the main part of the integument, may be at least 40%, with the exact figure varying according to the insect species.<sup>6</sup> Chitin provides a light but strong scaffold material which is associated with cuticle proteins that determine the mechanical properties of the cuticle itself.<sup>4</sup> Within the cuticle, there are two main chitin-containing layers with distinct structural features. These are the outer exocuticle and the inner endocuticle.<sup>7</sup>



(a) exocuticle



(b) endocuticle

**Figure 2:** Microstructure of lobster cuticle as shown by SEM.<sup>7</sup>

In the exocuticle (Figure 2) the mineralized chitin-proteins fibres are thin and arranged in parallel, whereas in the endocuticle (Figure 2) the chitin-protein fibres are stacked in horizontal



planes. The fibre direction (white arrows) rotates around the normal axis of the cuticle. Every stack of planes rotated  $180^\circ$  is referred to as one Bouligand layer.<sup>7</sup>

## 1.2. Role of chitin in insects

Chitin is also found in insect peritrophic matrices, where it provides an absorbent membrane between the food bolus and the midgut epithelium. This helps digestion and protects the midgut from mechanical damage and harmful organisms.<sup>8</sup> Insect peritrophic matrices are usually composed of between 3-13% chitin,<sup>9</sup> although studies on the tobacco hornworm *Manduca sexta* reported a chitin content of 40%.<sup>6</sup> The rate of matrix formation often varies depending on physiological conditions.<sup>9</sup> The peritrophic matrix itself is formed by the association of chitin microfibrils with highly hydrated proteoglycan material secreted by the insect gut cells, and the rest of the non-chitin content is a complex mixture of proteins and glycoproteins.<sup>8</sup> This includes proteins called peritrophins that bind to chitin microfibrils *via* multiple chitin-binding domains and also mediate binding to other glycoproteins.<sup>10</sup> In this way they may add significant mechanical strength to the peritrophic matrix.

Chitin polymers are cross-linked by hydrogen bonds between the amine and carbonyl groups, which usually leads to the formation of chitin microfibrils of  $\sim 3$  nm.<sup>11</sup> In peritrophic matrices however, the chitin microfibrils may be more than  $0.5\ \mu\text{m}$  in length with 10 or more of these single microfibrils associated together as parallel bundles.<sup>9</sup> Analysis by x-ray diffraction has shown that chitin in general is a polymorphic substance that occurs in three different crystalline modifications, termed  $\alpha$ -,  $\beta$ - and  $\gamma$ -chitin.<sup>7, 11</sup> The degree of hydration, the size of the unit cell and the number of chitin chains per cell, determine the polymorphic state of the chitin.<sup>11</sup> In  $\alpha$ -chitin, all chains are laid out in an anti-parallel orientation, whereas in the  $\beta$ -form the chains are arranged in a parallel fashion. In  $\gamma$ -chitin, sets of two parallel strands interchange with single antiparallel strands.<sup>4, 7</sup> All three polymorphic states of chitin are found in insects, while in fungal systems non-crystalline, transient states have also been reported.<sup>12</sup> In insects,  $\alpha$ -chitin is mostly found in chitinous cuticles,<sup>13</sup> as well as peritrophic matrices.<sup>9</sup>  $\beta$ -Chitin also occurs in peritrophic matrices, and is often found in cocoons, as is  $\gamma$ -chitin.<sup>10, 11</sup>

The physiochemical properties of the insect cuticle such as mechanical strength and stability<sup>13, 14</sup> rely upon the anti-parallel arrangement of  $\alpha$ -chitin chains. This allows tight packaging into chitin microfibrils, in which around 20 chitin units are held together by inter-chain hydrogen

bonds.<sup>9,11</sup> Insect body parts that are used to hold prey, and should therefore ideally be hard and strong also tend to be composed of  $\alpha$ -chitin; for instance, the grasping spines of the arrow worm *Sagitta* are made of pure  $\alpha$ -chitin,<sup>15</sup> although the pen of *Cephalopoda* is  $\beta$ -chitin. In general, flexible and softer chitinous structures such as peritrophic matrices or cocoons tend to contain more  $\beta$ - and  $\gamma$ -chitin.<sup>9</sup> In the  $\beta$ - and  $\gamma$ -forms, there are fewer inter-chain hydrogen bonds, an increased number of hydrogen bonds with water,<sup>17</sup> and overall the chains are less tightly packed.

The association of chitin with proteins and minerals plays a key role in providing properties of resistance and flexibility throughout the anatomy of arthropods.<sup>4</sup> Chitin-protein complexes are responsible for exoskeleton elements such as trachea, cuticle, stomodeum and the peripheral membrane of arthropods.<sup>18, 19</sup> The colouration of the cuticles has its origin from the binding of chitin with carotenoids.<sup>4</sup>

### **1.3. Chitin metabolism in insects and microorganisms**

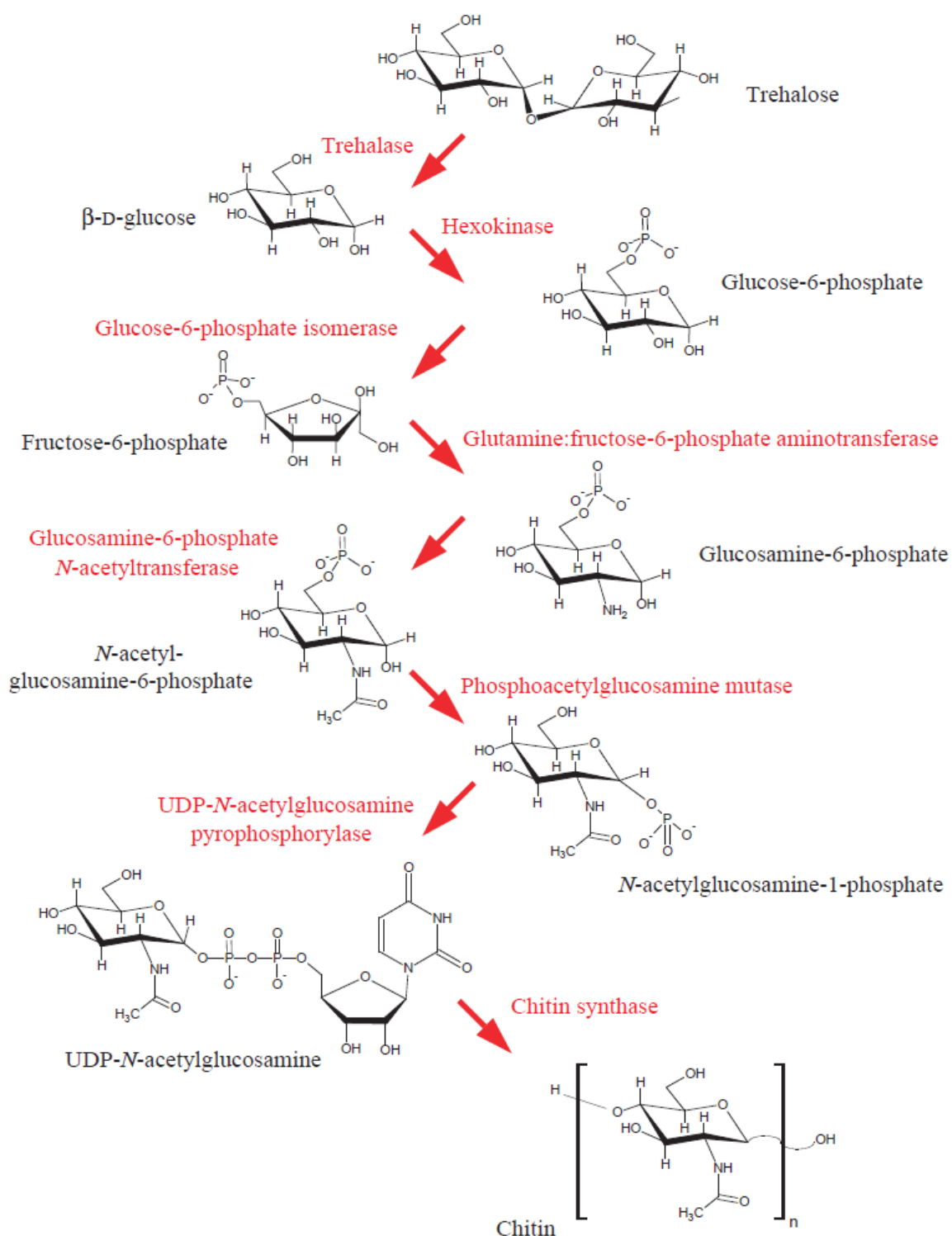
Insects need to periodically replace their old cuticle with a new and loose one during moulting (ecdysis).<sup>11</sup> The new synthesized cuticle can only be expanded when moulting is complete.<sup>5</sup> After ecdysis occurs, the old endocuticle is digested by proteases and chitinases.<sup>20, 21</sup> This means that insect growth and development depends on the capability to remodel chitinous structures,<sup>22</sup> and so insects must consistently synthesize and degrade chitin in a highly controlled manner to allow ecdysis and regeneration of the peritrophic matrices.<sup>9</sup>

Chitin metabolism plays a crucial role in the life cycle of metazoan and protozoan parasites that affect humans.<sup>23</sup> In some parasites chitin is a structural component of these organisms, whereas in others, parasites interact with the chitin-containing structure, which they come upon during development, *e.g.* *Entamoeba* parasites.<sup>24</sup> The eggshell and sheath integument of microfilarial worms contains chitin.<sup>3</sup> Also chitin is a component of the cyst wall of the intestinal pathogens *Entamoeba* and *Giardia*.<sup>25, 26</sup> These parasites secrete chitinases in order to modulate their chitinous structure. The fungal cell wall is also composed of chitin, which protects these organisms from the environment.<sup>2</sup>

## 1.4. Chitin biosynthesis

The biosynthetic pathway to chitin involves the conversion of glucose to uridine diphosphate *N*-acetylglucosamine (UDP-GlcNAc).<sup>5, 11</sup> It was suggested that seven enzymes catalyse the reactions in the synthesis of chitin, as shown in Figure 3.

The formation of glucosamine-6-phosphate occurs from glucose and is catalysed by hexokinase. Fructose-6-phosphate is obtained by the action of glucose-6-phosphate isomerase, which reacts with glutamine and glutamine transaminase to transfer an amino group from glutamine to fructose-6-phosphate to give glucosamine-6-phosphate. By the action of phosphoglucosamine transacetylase and acetyl coenzyme-A, acetylglucosamine-6-phosphate is obtained and then acetylglucosamine-1-phosphate is obtained by the action of phosphoacetylglucosamine mutase. Uridine diphosphate acetylglucosamine (UDPAG) is formed from uridine triphosphate and acetylglucosamine-1-phosphate, which is the substrate for chitin synthase.<sup>11</sup>



**Figure 3:** Biosynthesis of chitin in insects. Reproduced from Merzendorfer.<sup>5</sup>

Many subsequent studies conducted with preparations from various insects support the general pathway, which is shown in Figure 3. Chitin synthase (CS) converts cytosolic uridine diphosphoryl-*N*-acetylglucosamine (UDP-GlcNAc) to chitin. However, little is known about the final steps in chitin synthesis in any organism.<sup>5</sup>

The pathway from UDPGlcNAc to chitin was determined by using the cell-free extracts from the southern armyworm and also using microsomal fractions from the blue crab and the brine shrimp.<sup>5, 27</sup> UDPGlcNAc and primer chitin oligosaccharides are precursors utilized by the synthase enzyme for chitin. Several carbohydrates, besides glucose, serve as precursors for chitin, including trehalose, fructose, glucosamine and *N*-acetylglucosamine.<sup>5</sup> The common features of chitin synthases from yeast, fungi and insect are their localization on vesicular membranes and slight activation after treatment with proteases such as trypsin.<sup>28, 29</sup>

Chitin synthase activity is inhibited by UDP-GlcNAc analogues such as polyoxins and nikkomycin,<sup>5</sup> and seems to be confined to membrane-containing fractions.<sup>29</sup> Chitin synthase has been found in the membranes of Golgi complexes<sup>30</sup> and intracellular vesicles, and also in plasma membranes, which suggests that the enzyme follows an exocytotic pathway, accumulating in cytoplasmic organelles during its transport to the cell surface.<sup>28</sup> Analysis on the imaginal discs of the Indian mealmoth *Plodia interpunctella*, showed that chitin synthesis is inhibited with colchine and vinblastine.<sup>5</sup> In fungal systems, the activity of one chitin synthase isoform (CHS3p) has been shown to be associated with intracellular microvesicles, known as chitosomes.<sup>31</sup> Electron microscopy studies of the chitosomes showed that they synthesise chitin microfibrils that crystallize in the lumen of these vesicles.<sup>5</sup> Similar results were obtained when chitin synthase activity in crude extracts of the red flour beetle *Tribolium castaneum* was examined. Electron micrographs showed that the chitin synthase products were arranged in parallel microfibrils with a thickness from 10 nm to 80 nm.<sup>32</sup> These microfibrils were associated with other particles of 50-250 nm which could be regarded as “insect chitosomes”.<sup>30</sup> Currently, little is known about the role of such insect chitosomes in chitin synthesis, and studies on both the Brazilian skipper butterfly *Calpodetes ethlius* and the Australian sheep blowfly *Lucilia cuprina*<sup>29</sup> have shown an absence of these types of structures. Electron microscopy studies instead indicated areas at the tips of microvilli from the epidermal cells to the clusters of chitin synthesising enzymes.<sup>29</sup>

## 2. Chitinases and their classification

The hydrolysis of chitin is catalysed by chitinases which convert the carbohydrate polymer into simple mono- and oligosaccharides. Chitinases can be classified into two different groups of enzymes - endochitinases and exochitinases – according to where they act on the polymer

chain.<sup>11</sup> Exochitinases act on the non-reducing end of the chitin polymer and be further divided into two different groups of enzymes. These are chitobiosidases, which progressively cleave off diacetylchitobiose units, and  $\beta$ -(1,4)-acetylglucosaminidases (NAGase), which cleave off only terminal, non-reducing *N*-acetylglucosamine (NAG) units, to generate NAG monomers.<sup>33</sup> Endochitinases in contrast, cleave random  $\beta$ -(1,4)-glycosidic bonds within the chitin polymer, giving a variety of low molecular weight NAG oligomers, such as diacetylchitobiose, as well as NAG trimers and tetramers.<sup>33-35</sup>

Chitinases are classified into Family 18 and 19 glycoside hydrolases (GH) according to their amino acid sequence similarity.<sup>36</sup> Members of the two distinct families differ both in their three dimensional structures and also the mechanisms by which they catalyse chitin hydrolysis.<sup>37, 38</sup> Family 18 chitinases have catalytic domains with triosephosphate isomerase (TIM-barrel) folds,<sup>39</sup> with a conserved motif of DxDxE residues, and hydrolyse chitin by a substrate-assisted mechanism.<sup>40-44</sup> On the other hand, the three dimensional structures of Family 19 chitinases have a significant degree of  $\alpha$ -helicity, and chitin hydrolysis is catalysed via a single displacement mechanism.<sup>45</sup> Family 18 contains chitinases from mammals, insects, plants, nematodes, fungi and bacteria, whereas Family 19 chitinases are only found in plants and *Streptomyces* species.<sup>46</sup> Family 18 chitinases have been well characterised, with information available on their three-dimensional (3-D) protein structures and their reaction mechanisms. The catalytic domain of known Family 18 chitinases consists of an  $(\alpha/\beta)_8$  barrel fold with a long deep substrate-binding cleft containing six binding subsites -4, -3, -2, -1, +1 and +2, with the cleavage site located between subsites -1 and +1.<sup>47</sup> Based on crystal structures, the catalytic reaction of Family 18 chitinases takes place through a substrate-assisted, double displacement mechanism with a geometrically deformed oxazolinium intermediate.<sup>44</sup> This has been demonstrated in structural analysis of hevamine and *Serratia marcescens* chitinase A. In contrast, the catalytic domain of Family 19 chitinases has a high  $\alpha$ -helical content and has structural similarity, including a conserved core, with chitosanase and lysozyme.<sup>46, 48</sup>

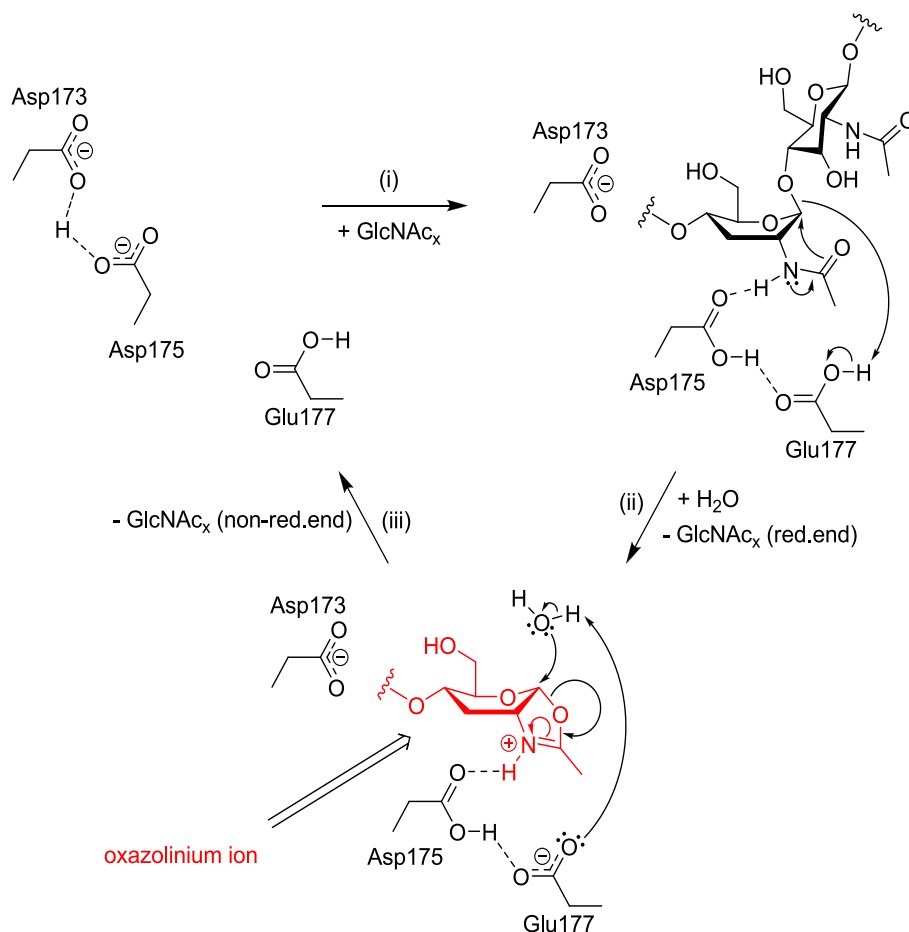
In addition to their 3D structure, chitinases of the two families show several important differences in their biological properties. For example, Family 18 chitinases hydrolyse the glycosidic bond with retention of the anomeric configuration,<sup>49</sup> whereas Family 19 chitinases hydrolyse with inversion.<sup>45, 50</sup> Family 18 are sensitive to allosamidin, but Family 19 from higher plants has been shown to be insensitive. Family 18 chitinases hydrolyse also GlcNAc-GlcNAc and GlcNAc-GlcN linkages, whereas Family 19 chitinases cleave GlcNAc-GlcNAc and GlcN-GlcNAc.<sup>48</sup> These differences are common between all members of the two families and arise

from the differences in their catalytic mechanisms. Substrate-assisted catalysis is the most widely accepted model for the catalytic mechanism of Family 18 chitinases, whereas a general acid and base mechanism has been suggested for the catalytic mechanism of Family 19 chitinases.<sup>50, 51</sup>

## 2.1. Family 18 chitinases: structural biology and catalytic mechanism

The reaction mechanism of Family 18 chitinases is well established. Chitooligosaccharides bind to the active site groove of Family 18 chitinases with the *N*-acetylglucosamine (GlcNAc) units stacking on solvent-exposed hydrophobic residues, with the -1 sugar assuming a boat conformation, as outlined in Scheme 1 for *Aspergillus fumigatus* chitinase B1 (AfChiB1), a typical bacterial-type chitinase. In the Family 18 chitinases, the amino-acid motif DXEDXE which is involved in catalysis is strongly conserved.<sup>41, 44, 49, 52</sup> Chitin binding also results in a reorientation of the Asp-175 side chain, replacing its hydrogen bonds to the carboxyl group of the catalytic acid Glu-177 and the -1 sugar C2 acetamido group of the substrate. Nucleophilic attack of the carbonyl oxygen of the acetamido group on the anomeric C1 carbon of the same pyranose ring occurs concurrently with protonation of the glycosidic oxygen by the catalytic acid, generating the leaving group from the reducing end of chitin as well as an oxazolinium ion intermediate (step (ii), Scheme 1).<sup>44, 45, 52</sup> Family 18 enzymes thus catalyse hydrolysis through the “substrate-assisted” mechanism, in which the essential steps are the generation of the oxazolinium intermediate by attack on the polymer of its own *N*-acetamido group and the retention of the  $\beta$ -glycosidic configuration after the glycosidic cleavage.

Like all TIM-barrel enzymes, in the Family 18 chitinases, the two catalytic residues Asp and Glu are located just beyond the carboxyl-ends of two of the eight  $\beta$ -strands making the  $(\alpha/\beta)_8$  barrel.<sup>53</sup> Thus evolution has led to substrate specificity for hydrolysis of the carbohydrate structure. For example, endochitinases hydrolyze a reducing end GlcNAc, while in contrast, exochitinases cleave disaccharides from the non-reducing ends of the soluble chitin oligosaccharides.<sup>54</sup>



**Scheme 1.** The catalytic cycle of Family18 exo-chitinases.

Arrows indicate electron transfer of the subsequent step, while dashed lines represent hydrogen bonds. (i) Binding of GlcNAc; (ii) cleavage of GlcNAc releasing product from the reducing end; (iii) release of product from non-reducing end. This figure is based on reaction schemes from Tews *et al.*<sup>44</sup> and van Aalten *et al.*<sup>40</sup> This reaction results in the retention of the stereochemistry at the anomeric carbon of the product, in contrast to the inversion mechanism of Family 19 chitinase. The soluble products of this catalytic mechanism are chitotetraose, chitobiose. These steps differ from the classical retaining mechanism of glycosyl hydrolases such as lysozyme, cellulose and endo-1,3-1,4- $\beta$ -D-glucan-4-glucanohydrolase that use a carbohydrate side chain as the nucleophile.

## 2. 2. Chitinases: occurrence and regulation

Chitinases play a role in the life cycle of many organisms including bacteria, fungi, plants, insects and animals. In insects and crustaceans, chitinases participate in the moulting process to



renew the cuticle,<sup>11</sup> while chitinases from bacteria and fungi help to maintain a balance between the large amount of carbon and nitrogen trapped in the biomass as insoluble chitin in nature.<sup>55</sup> In fungi, chitinases are needed for cell separation,<sup>56</sup> and chitinases from some plants play a protective role against fungal pathogens.<sup>57</sup> The human chitotriosidase may also be important as a defence against chitin-containing pathogens such as *Candida albicans*.<sup>58</sup>

### **2.2.1. Importance of chitinases in metabolism**

The roles of chitinases in organisms are diverse. In fungi, chitinases are thought to have autolytic, nutritional and morphogenetic roles.<sup>56, 59</sup> Chitinases in bacteria have been shown to play a role in the digestion of chitin for utilization as a carbon and energy source and recycling chitin in nature.<sup>60</sup> In insects, chitinases are associated with sclerotized proteins to allow growth and development.<sup>5</sup> Plant chitinases are involved in defence and development.<sup>61</sup> In the human genome, chitinases have been suggested to play a role in defence against chitinous human pathogens.<sup>62</sup>

### **2.2.2. Chitinases in insects**

It is known that arthropods consistently synthesize and degrade chitin using proteases and chitinases to allow ecdysis and regeneration of the peritrophic matrices.<sup>5, 9</sup>

Insect chitinases belong to the sub-class of Family 18 chitinases and share a high degree of amino acid similarity.<sup>38</sup> The most remarkable difference among chitinases from different kingdoms is the large range in their sizes. The smallest chitinases consist primarily of a catalytic domain, whereas the larger chitinases often are organized into multiple domains with distinct functions. Insect chitinases obey these rules, thus they have theoretical molecular masses ranging between 40 kDa and 85 kDa. The basic structure consists of three different domains: a catalytic domain possessing one or more chitin-binding domains which can be either C-terminal or N-terminal, a binding domain with one or more fibronectin type 3, a PEST-like region, enriched in proline, glutamate, serine and threonine and a cysteine-rich region.<sup>22</sup> All these domains play a role in glycosylation and protein turnover.

Chitinases also differ in their pH optima (pH 4-8) and isoelectric points (IP 5-7). This contributes to their catalytic parameters, affinity for chitin and stability.<sup>5</sup> The tobacco hornworm (*Manduca sexta*) has an insect chitinase responsible for the moulting process.<sup>22</sup> This enzyme

possesses multiple domains, including an *N*-terminal catalytic domain, a middle serine/threonine and a *C*-terminal cysteine-rich domain. In the moulting secretions, three different forms of this protein with sizes 50 kDa, 62 kDa, 85 kDa have been detected. They all are enzymatically active and cross-related immunologically.<sup>11</sup> In all insect chitinases, the PEST-like region acts as a signal peptide for the glycopolymer degradation.<sup>22</sup> The signal peptide transfers secretion of the chitinase to the endoplasmic reticulum, which is released by a signal peptidase after the protein has been transported across the membrane.<sup>63</sup>

It is known that, as similarities of amino acid sequences are present in different glycosyl hydrolase families, the catalytic mechanism within each family is highly conserved.<sup>38, 39</sup> Members of sequence-related families will have similar folds and this can direct the choice of appropriate search models for molecular replacement and gives potential for homology modelling of related sequences.<sup>53</sup> The catalytic domain of Family 18 chitinases has a  $(\alpha/\beta)_8$  TIM-barrel which consists of a cylindrical, barrel-like structure made from eight parallel  $\beta$ -strands, that are alternately connected with eight  $\alpha$ -helices.<sup>64</sup> A full crystal structure of an insect chitinase is not yet available but homology modelling using crystal structures of bacterial and plant chitinases has provided three-dimensional models of the catalytic domain.<sup>65</sup> The *Manduca* chitinase showed a partial signature of the  $(\alpha/\beta)_8$  folding and a crystal structure showed  $\beta$ -sheets and four complete and several incomplete  $\alpha$ -helices.<sup>22</sup> Like some fungal chitinases, the chitinases found in insect moulting fluids are glycosylated. Additionally, the PEST-like region of *Manduca* chitinase is mostly modified by *O*-glycosylation.<sup>66</sup>

Insect chitinases contain a *C*-terminal chitin-binding region, whose three-dimensional structure includes a six-cysteine motif that is also seen in nematode chitinases.<sup>67</sup> The cysteine motif targets the chitinase towards its substrate and so helps facilitate chitin hydrolysis.<sup>68</sup> This structural feature also occurs in peritrophic matrix proteins, as well as in receptors and other proteins that are involved in cellular adhesion.<sup>22, 68</sup>

Enzymatic glycosidase reactions normally require the presence of two catalytic groups which in most cases have been observed to be either aspartic acid or glutamate residues.<sup>39</sup> The Family 18 chitinases utilise conserved acidic amino acids, in their hydrolysis mechanism, similar to those observed in other glycosidases.<sup>43</sup> Chitinases from insects have the conserved active site sequence YDFDGLDLDWEYP, which identifies them as Family 18 chitinases,<sup>69-71</sup> and this is

confirmed by the fact that mutation of the glutamic acid residue site leads to a loss of enzymatic activity.<sup>72</sup>

Many of the sequence-related Family 18 chitinases from insects are polyspecific, which means they contain enzymes of different substrate specificities, suggesting an evolutionary divergence. Chitin degradation in *M. sexta* is carried out by chitinase and  $\beta$ -*N*-acetylglucosaminidase,<sup>20</sup> while the chitinases from *Manduca*, *Bombyx* and fall webworm moth *Hyphantria cunea* display the three basic domains (a catalytic domain, chitin-binding, PEST-like region and cysteine-rich region).<sup>66, 73</sup> Some other chitinases lack the PEST-like or the typical chitin-binding regions.<sup>74</sup> Enzymes with similar specificities are sometimes found in the different chitinase families which raises the possibilities of convergent evolution. In *Aedes*, for example, chitinases are encoded by two different genes. Nucleotide sequencing has revealed that one of the genes encodes three separate chitinases, each containing a catalytic and also a chitin-binding domain.<sup>71</sup> The beetle *Tenebrio molitor* encodes 5 chitinases *TmChit5*, which also displays an unusual structure, since 5 chitinases contain units of approximately 480 amino acids that are separated by PEST-like, chitin-binding and mucin-like domains.<sup>75</sup>

Chitin in arthropods plays a key role in development and growth, and so its hydrolysis is as tightly regulated as its synthesis.<sup>11</sup> Monitored transcription or enzyme activities have shown that the expression or activity of the integument chitinases is restricted to periods of moult and pupation,<sup>11</sup> whereas gut chitinases are expressed after feeding.<sup>76</sup> Interestingly, chitinase expression in the moulting fluid appears after  $\beta$ -*N*-acetylglucosaminidase expression.<sup>66</sup> In epidermal cells, 20-hydroxyecdysone stimulates the secretion of the molting fluid containing chitinases and proteases, indicating that this hormone may also be involved in the regulation of chitinase expression and activity. For example, the chitinases of *Bombyx* and *Manduca* are induced at high haemolymph levels of 20-hydroxyecdysone, while  $\beta$ -*N*-acetylglucosaminidases are induced at low levels of this ecdysteroid.<sup>77</sup> Consistent with this,  $\beta$ -*N*-acetylglucosaminidase expression was found to start before chitinase expression in the larvae of the yellow fever mosquito, *Aedes aegypti*.<sup>76</sup>

Cuticles from arthropods are associated with proteins such as resillin, a glycine-proline rich protein that confers rigidity properties.<sup>4</sup> In order for the cuticle to be degraded by chitinases, proteases are needed, not only to break down this structural material directly, but also to cleave inactive chitinase precursors and release the active enzymes, as suggested for *Manduca* or

*Tenebrio* chitinases. This kind of proteolytic activation of chitinases may also occur in insect gut systems, so in the case of blood-sucking insects, the activities of gut chitinase and  $\beta$ -*N*-acetylglucosaminidases are found to rise shortly after feeding.<sup>76</sup> For instance, the gut-specific chitinase of *Anopheles* needs to be trypsinized in order to develop chitinase activity.<sup>78</sup> The malaria parasite *Plasmodium falciparum* also makes use of the protease-rich environment in the mid-gut of the mosquito host in order to induce the enzymatic activity of its own chitinase which allows it to penetrate the peritrophic matrix.<sup>79</sup>

### 2.2.3. Chitinases in plants

Plant chitinases are divided into classes I-V within which classes III and V belong to Family 18 and classes I, II and IV belong to Family 19 chitinases.<sup>48</sup> Three classes of plant chitinase have been identified based on the primary structure. These classes I, II and IV chitinases all have a signal peptide domain and possess similarities in the catalytic domain.<sup>80</sup> Class I chitinases are enzymes with *N*-terminal cysteine-rich domains linked by a proline/glycine-rich region (signal peptide region) to the catalytic domain. The cysteine-rich domain is also called a wheat-germ agglutinin domain, which is important for chitin binding, but not for catalytic activity.<sup>81</sup> Most of the class I chitinases contain a *C*-terminal signal peptide that is essential for targeting the plant cell vacuole.<sup>81</sup> Class II chitinases, mainly found in dicotyledons, lack the *N*-terminal cysteine-rich domain and *C*-terminal signal peptide, indicating that these chitinases do not bind chitin and they are secreted to be apoplastic.<sup>82</sup> Class IV are also found in dicotyledons, contain a cysteine-rich domain and a conserved main structure which resemble to those of class I chitinases but are significantly smaller due to four deletions.<sup>57, 81</sup> Class III are mainly found in plants and fungi; they possess bifunctional lysozyme/chitinase activity, such as the enzyme of *Havea brasiliensis*.<sup>83</sup>

Plant chitinases are classified as pathogenesis-related proteins (PR), which are expressed upon attacks by viruses, bacteria and fungi as well as pest attack.<sup>57</sup> It has been found that PR proteins are induced by various stress factors such as drought, salinity, wounding, heavy metals in their environment, endogenous and exogenous elicitor treatment and plant growth regulators.<sup>84-86</sup> The induction of chitinase in plants is often co-ordinated with the induction of specific  $\beta$ -1,3-glucanases and other PR proteins.

The role of chitinases in plants is as a defence mechanism against chitin-containing organisms, such as insects and fungi.<sup>87</sup> A plant pathogenic fungus *Rhizoctonia solani*, is known to cause serious plant losses, by attacking primarily the roots and lower stems and its hosts are herbaceous plants.<sup>88</sup> The levels of chitinases are upregulated in rice cultivars when there is an attack by the pathogen *Rhizoctonia solani*. Chitinases in plants are associated with  $\beta$ -1,3-glucanases, which inhibit the growth of chitin-containing fungi by causing decomposition of hyphal tips in juvenile cells containing exposed nascent chitin chains that are accessible to hydrolysis. The chitin layer in the mature cell wall is cross-linked with polysaccharides forming mixed chitin-glucan fibres and is therefore, relatively inaccessible to hydrolysis.<sup>89</sup>

Plant cellular and secretory chitinases are suggested to have different roles in defence reactions. Apoplastic chitinases are considered to be part of an early, induced response as these chitinases act directly by blocking the growth of the hyphae invading the intracellular space.<sup>90</sup> During the infection process, cellular chitinases are released when hyphae penetrate and affect cell integrity.<sup>57</sup> Class I vacuolar chitinases are strongly induced by jasmonate/ethylene pathways.<sup>61</sup> Both class I and class II chitinases are induced as part of the local hypersensitive response. On the other hand, class II chitinases are induced as a response to the pathogen attack.<sup>91</sup> Cellular chitinases inhibit pathogen growth more efficiently, due to their rapid mutation occurring in the active site. This faster mutation allows for following of evolutionary changes in fungal cell structure, whereas the active site of apoplastic chitinases is more conserved and most of the evolutionary changes occur in the non-catalytic domain.<sup>61</sup>

Most saprophytic pathogens live and survive in the soil and attack the parts of their host that reside there. Consequently chitinase mRNAs accumulate at elevated levels in seeds in different plant species. Seed chitinases act in a synergistic way with ribosome-inactivating proteins and  $\beta$ -glucanase proteins to provide antifungal properties.<sup>92</sup> The role of chitinases in plant defence against fungi has been established with genetic transformation experiments. The transgenic plants have been produced using chitinase genes of diverse classes and by employing a range of donor and recipient host plant species.<sup>93</sup> Transgenic tobacco plants constitutively expressing a bean class I chitinase gene showed an enhanced resistance to the fungal root pathogen *Rhizoctonia solani*.<sup>94</sup> A partial resistance against *Fusarium* species in tomato was demonstrated by simultaneous expression of a class I chitinase and a class I  $\beta$ -1,3-glucanase gene from tobacco. Transgenic plants that overexpress either chitinases singly or chitinase in combination with other antifungal proteins have also been developed.

Chitinases are induced by a range of different abiotic stresses such as osmotic, salt, cold, wounding and heavy metal stress.<sup>87</sup> For instance, to heal wounds, Class I chitinases in fig tree latex are induced. *Brassica juncea* chitinase (BjCHI) expression is also highly induced by methyl jasmonate, wounding, NaCl and polyethylene glycol stresses,<sup>95</sup> while class I chitinases from tall fescue turf grass are overexpressed by dehydration and ethylene.<sup>96</sup> Additionally, chitinases have roles in protecting against the toxicity of heavy metals, such as lead, arsenic and cadmium that occurs in faba beans, barley, maize and soybean.<sup>97</sup>

Chitinase activity is induced in *Arabidopsis* by UV light, heat, shock and wounding.<sup>90</sup> While class I and IV chitinase genes were expressed in most organs of *Arabidopsis* under normal growth conditions, class III genes were expressed exclusively when the plants were exposed to environmental stresses, notably salt and wound stresses.<sup>98</sup>

Chitinases have been shown to be expressed during cold hardening in rye, indicating their antifreeze activity and the ability to inhibit and modify ice crystal growth in plant apoplasts. In *Cynodon* species, a class II chitinase gene was induced during cold conditions and dehydration.<sup>99</sup> Cold-responsive extracellular chitinase was also found to have antifreeze activity in bromegrass cell cultures. Chitinases are also expressed under drought conditions in wheat. Transgenic tobacco plants that overexpressed chitinase from *Trichoderma harzianum* also conferred resistance against salinity and heavy metals.<sup>100</sup>

Chitinases also regulate processes of growth and development by generating or degrading signal molecules, as during nodulating processes, where the bacterial lipochitooligosaccharides (Nod factors) are degraded by chitinases.<sup>61</sup>

#### **2.2.4. Role of chitinases in other microorganisms (bacteria, nematodes and fungi)**

The fungal human pathogen *Candida albicans* utilizes chitinases during cell division to separate daughter cells.<sup>101</sup> The eggs of the nematode gastrointestinal parasite of mice, *Helimosomoides polygyrus*, express increasing levels of chitinases with age up to hatching and *Entamoeba invadens*, a model for pathogenic amoebic encystations, produces chitinases during encystation.<sup>102</sup> Vertebrates are therefore exposed to chitin through ingested food or when infected with nematodes or fungi.

Chitinases are present in various non-chitin containing organisms, where chitin is used as a source of nutrients for growth. Some bacteria produce chitinases for processing and digestion of GlcNAc-containing macromolecules found in nature, which are sources of their nutrients.<sup>60</sup> *Streptomyces* species are soil bacteria and some of the main decomposers of chitin. They use chitin as carbon and nitrogen sources and so play a major role in the turnover of chitin in natural ecosystems. Baculoviruses, such as *Bombyx mori* nucleopolyhedrovirus, express chitinases and cathepsin, which cause dissolution of the insect cell host, resulting in the complete loss of all insect tissues, permitting mature viruses to escape into the environment and promoting horizontal virus transmission.<sup>103</sup> Malaria parasites (ookinetes) digest the peritrophic membrane in the mosquito midgut during penetration.<sup>104</sup> Parasites also degrade chitin-containing host organisms in order to facilitate their further growth and transmission. *Leishmania* parasites and those causing Chagas disease also do not contain chitin but secrete chitinases to degrade the internal chitin-containing structures of their insect vectors.<sup>79</sup> Chitinases in the reptilian parasite *Entamoeba invadens* encyst parasites within a cell wall, allowing resistance to environmental conditions.<sup>24</sup> *Serratia marcescens* chitinases digest a chitinous peritrophic membrane, leading to the release of *N*-acetylglucosamine and fragments of the membrane.<sup>36</sup>

### 2.2.5. Chitinases in Humans

Despite the absence of endogenous chitin, humans express two active chitinases, designated chitotriosidase (CHIT1),<sup>62, 105</sup> and a second, given its acidic pH optimum, named acid mammalian chitinase (AMCase).<sup>105</sup> AMCase is expressed mainly in salivary glands and in the stomach,<sup>106</sup> whereas CHIT1 is expressed by phagocytic cells in humans and extremely high levels are seen in lysosomal storage diseases, such as Gaucher's disease.<sup>107</sup> Both are endo- $\beta$ -1,4-*N*-acetylglucosaminidases that are believed to be involved in food digestion and the immune response.<sup>108</sup> Zhu *et al.* showed that AMCase expression was increased in the lungs of ovalbumin-sensitized mice compared with the control group, suggesting a key role of this enzyme in asthma pathophysiology.<sup>109</sup> In this asthma model, AMCase does not directly induce Th2 cytokine response but mediates the effector response of interleukin-13 (a cytokine produced by Th2 cells). AMCase possesses the ability to exacerbate local inflammation by the activation of the IL-13 pathway and facilitating the production of chemical mediators, suggesting that AMCase inhibitors are a potential target in Th2-mediated inflammation.<sup>1</sup> More recently studies have shown a crucial role of AMCase in ocular inflammation.<sup>110</sup>

Chitotriosidase was first discovered in the plasma of patients suffering from Gaucher's disorder.<sup>62</sup> The protein activity was strongly elevated because of its high production and secretion by the lipid-laden macrophages that accumulate in these patients. Subsequently it has been purified from the spleen of a Gaucher patient and its cDNA was cloned from a human macrophage cDNA library. Analysis revealed that the enzyme belongs to the Family 18 chitinases and shares significant sequence identity with chitinases from non-mammalian species. Chitotriosidase is known to exist in two forms, a 50-kDa protein and a 39-kDa enzyme that are produced therefore by proteolytic processing. The 50-kDa protein exhibits a C-terminal chitin-binding region and a hinge region and the 39-kDa enzyme consists of an N-terminal domain that has chitinase activity.<sup>62</sup>

The established antifungal action of chitinases in plants and the tightly regulated expression of chitotriosidase in phagocytes suggest that this protein might fulfil a role in the degradation of chitin-containing pathogens.<sup>58</sup> Chitotriosidase is also expressed in human polymorphonuclear neutrophils (PMNs) and macrophages.<sup>62</sup> The enzyme is selectively expressed and released upon specific stimuli by human PMNs as well as macrophages. In PMNs the protein is found to be stored in the specific granules from which it is released by appropriate stimuli.<sup>62</sup> Studies *in vivo* have showed that chitotriosidase inhibits growth of *C. neoformans*, causes hyphal tip lysis in *Mucor rouxii* and prevents *Candida albicans* switching from the yeast to a hyphal morphology, which is important for pathogenicity and virulence. On the other hand, studies *in vitro* carried out with a model of mice affected with neutropenia-imposed infections from *C. albicans* and *Aspergillus fumigatus* have shown that recombinant human chitotriosidase improves the survival in these models. Preliminary investigations have showed that chitotriosidase activity was overexpressed in plasma of neonates with *C. albicans* infections. These results showed that chitotriosidase possesses an activity or anti-fungal role similar to chitinases in plants.<sup>58</sup> Additionally, recombinant chitotriosidase might be used to treat life-threatening fungal infections and Gaucher disease (a glycolipidic disorder). Chitotriosidase was also found to be at high levels in plasma of African children infected with acute *Plasmodium falciparum* malaria. As it is known that two active chitinases are expressed in the human genome, with AMCase implicated in asthma<sup>109</sup> and chitotriosidase being shown to target chitin-containing pathogens,<sup>58</sup> this opens a route to design new drugs for fungal diseases.

Interestingly, human cartilage family glycoprotein 39 (HCgp39/YKL-40), a chitin-binding lectin, has sequence homology with Family 18 chitinases but lacks enzymatic activity, due to a



critical amino acid replacement in the active site.<sup>111</sup> YKL-40 is present in specific granules of human neutrophils.<sup>112</sup> Many of the chitinase family proteins are expressed in macrophages and epithelial cells of the lung and digestive tracts, consisting of the body's first line of defence against exogenous chitin-containing pathogens. It has been shown in humans that levels of YKL-40/BRP39 in sera and lung tissues were significantly correlated with asthma development and even severity of the disease.<sup>113</sup> A significant role of these proteins in the generation of intestinal bowel disease (IBD) as an active mediator in acute colitis has also been suggested.<sup>114</sup>

### 2.3. Targeting chitinases

Since chitin metabolism is crucial for fungal<sup>2</sup> and arthropod development,<sup>5</sup> inhibition or deregulation of chitinases are important objectives for the development of fungicides<sup>115</sup> and also for the treatment of inflammatory diseases such as asthma and neglected tropical diseases.

Chitin metabolism plays a crucial role in the life cycle of parasites. Chitinases allow parasites to remodel their structures, develop and grow. The expression of stage-specific chitinases in parasites represents a potential target for blocking the development and the transmission of these parasites, particularly as chitin polymers are absent in vertebrates.<sup>60</sup>

The strategies for developing chitinase inhibitors would be dependent on the parasites. For some parasites, a chitinase inhibitor can act as a drug; for some others a vaccine can be prepared against the vertebrate stage of the parasite, in some other cases, a transmission-blocking vaccine against the vector would be suitable.

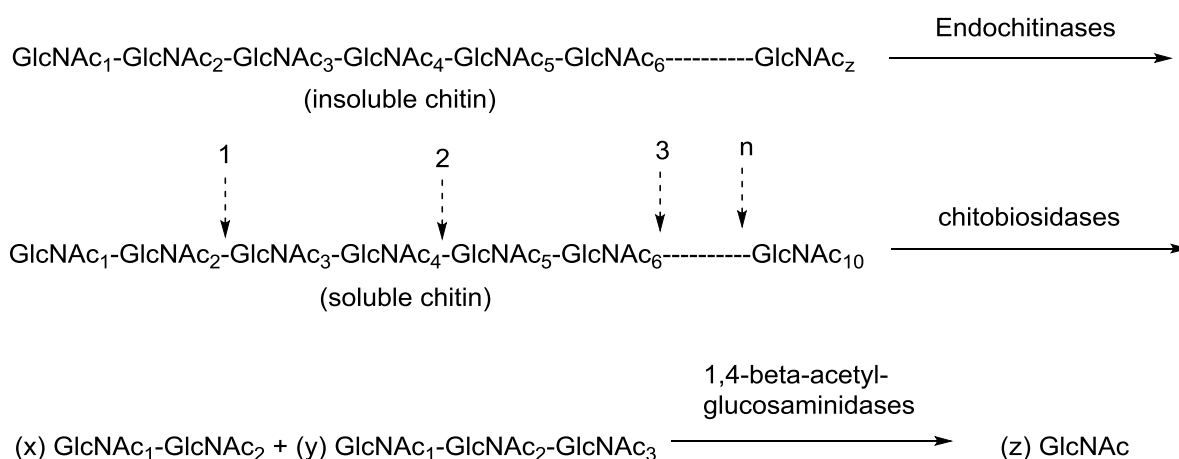
Various chitinase genes from human parasites have been identified, such as filarial nematodes (*Brugia malayi*, *Wuchereria bancrofti* and *Onchocerca volvulus*).<sup>116</sup> *Plasmodium gallicenaum*, an avian parasite similar to the human malaria parasite *P. falciparum*, has also been identified.

The potent chitinase inhibitor allosamidin delays the cyst formation of *Entamoeba invadens*, so allosamidin acts as a drug.<sup>24</sup> Chitinase antibodies were detected in sera of immunologically protected individuals with recombinant *B.malayi* chitinase and antigen of the monoclonal antibody MF1. This suggests that interfering with chitinase activity would block the establishment of filarial infection. Therefore chitinase inhibitors would be prophylactic agents

in this case. The hatching of filarial eggs and separation of mature nematodes in humans also suggests that a chitinase inhibitor could be used as a drug against microfilarial infection.<sup>74</sup>

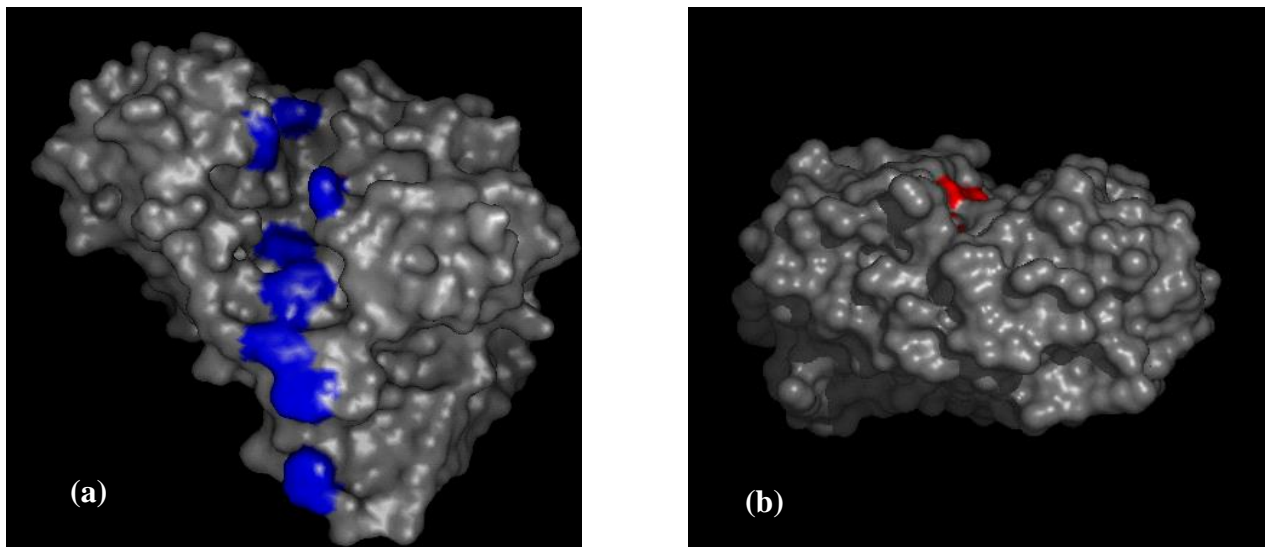
## 2.4. Bacterial and plant chitinases classes

There are two sub-classifications amongst Family 18 chitinases, which are bacterial-type and plant-type.<sup>47</sup> The plant-type Family 18 enzymes are endo-chitinases (EC 3.2.1.14) and cleave chitin randomly at  $\beta$ -(1,4)-glycosidic bonds in internal sites, generating soluble low-molecular-mass multimers of GlcNAc, giving oligosaccharide products of varying length such as chitotetraose, chitotriose and diacetylchitobiose. The bacterial-type enzymes are usually exo-chitinases (EC 3.2.1.19), meaning that specific  $\beta$ -(1,4)-glycosidic bonds are cleaved along the oligosaccharide chain such that di-acetylchitobiose (GlcNAc<sub>2</sub>) or acetylchitotriose (GlcNAc<sub>3</sub>) is progressively released from the non-reducing end of chitin. 1,4- $\beta$ -Acetyl-glucosaminidases (EC 3.2.1.30) then cleave the oligomeric products of endochitinases and chitobiosidases generating monomers of GlcNAc, as outlined in Scheme 2.<sup>47, 53</sup>



**Scheme 2.** Degradation of chitin by bacterial chitin-processing enzymes, adapted from Aronson *et al.*<sup>53</sup>

A key difference between the bacterial and the plant-type enzymes is that in the former there is a well-defined active site groove, lined with conserved aromatic residues to which chitin binds. The plant-type Family 18 chitinases, however, are generally smaller and usually contain less clearly identifiable sub-sites compared to the bacterial type-enzymes. This is an important consideration in the design of inhibitors for the two sub-classes of enzymes.



**Figure 4:** (a) The active site of a bacterial-type *exo*-chitinase, *Serratia marcescens* chitinase B *SmChiB*, (b) a plant-type *endo*-chitinase, hevamine, (Image courtesy of Prof. D. M. F. van Aalten, University of Dundee).

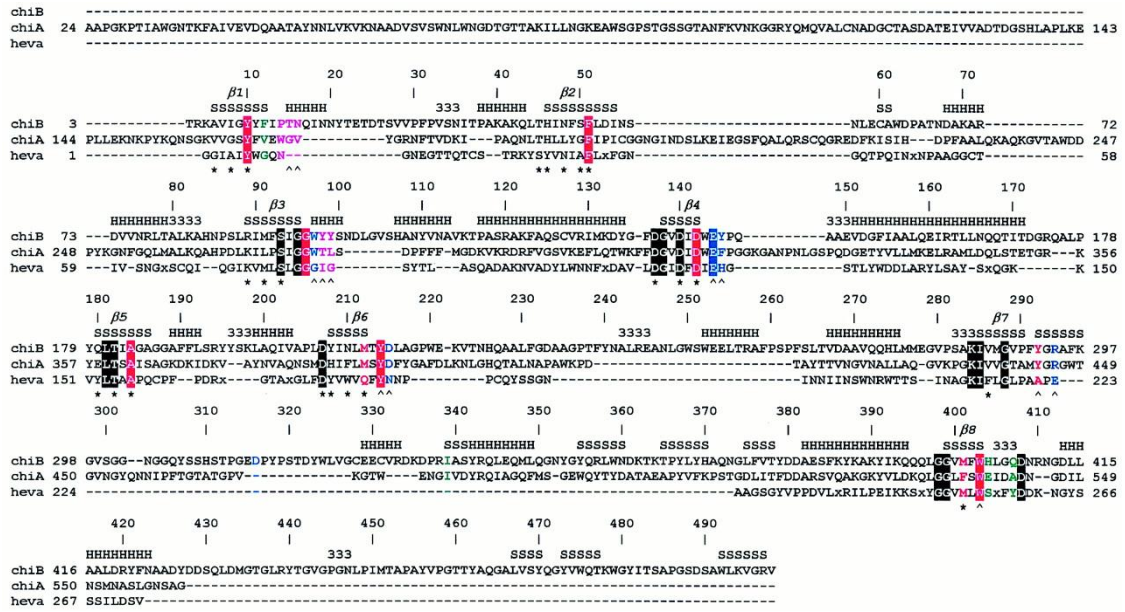
The bacterial type chitinase B1 from *AfChiB1* is the most secreted protein by this saprophytic pathogen. It possesses a  $(\beta/\alpha)_8$  barrel fold but lacks helix  $\alpha 1$  similar to other proteins from Family 18 glycoside hydrolases. Asp-175 and Glu-177 at the end of  $\beta 4$  represent the signature of the Family 18 chitinases, which is the DxE motif, with the glutamate residue being the catalytic acid. The active site groove is lined by several solvent-exposed aromatic residues, including Trp-137 and Phe-251. The crystal structure of an inhibitor, argifin, in complex with *AfChiB1* indicates that a *N*-methylphenylalanine is inserted tightly between these two hydrophobic residues, with a guanidinyurea moiety stacking on the amine of the oxazolinium ion intermediate and on the conserved Trp-384 and interacting with Asp-175 and Glu-177.<sup>117</sup> In plant-type chitinases such as *AfChiA1* which have the  $(\beta/\alpha)_8$  fold, most of the loops connecting the helices and strands are relatively short. Helix  $\alpha 8$  is interrupted by a minimal  $\beta$  sheet comprising strands  $\beta 9$  and  $\beta 10$ . The active site is defined by the very short  $\beta/\alpha$ -loops and lies at the *C*-terminal opening of the  $\beta$ -barrel.<sup>118</sup> In contrast, *AfChiB1* contains larger  $\beta/\alpha$ -loops including an entire  $\alpha/\beta$  domain that forms a shallow active site groove, lined by several solvent-exposed aromatic residues such as Trp-137.<sup>118</sup> The differences in the construction of the active site cleft are reflected in different affinities for the substrate.

The chitinase *SmChiA*, a Family 18 chitinase from the Gram-negative soil bacterium *Serratia marcescens* is the most studied bacterial chitinase, on account of its physio-biological properties. The enzyme has optimum activity at pH 4.5-7.0. The main product of chitin cleavage

is diacetylchitobiose and free NAG at higher enzyme or substrate concentrations. The enzyme acts as an exochitinase and cleaves the oligosaccharide from the non-reducing end of chitin.<sup>54</sup> A Michaelis constant ( $K_m$ ) of 4.4 mM (expressed in NAG equivalents) was reported for the enzyme, which was shown to be highly specific for chitin, over all other yeast cell-wall components.<sup>54</sup> The structure of *SmChiA* consists of three domains. The amino-terminal domain (residues 24–137), which consists only of  $\beta$ -strands, connects through a hinge region (residues 138–158) to the main  $\alpha/\beta$ -barrel domain (residues 159–442 and 517–563). The third domain, which has an  $\alpha + \beta$  fold, is formed by an insertion in the barrel motif (residues 443–516), as shown in Figure 5.<sup>119</sup>

Microbial chitinases, such as *SmChiA*, are important agents in resistance against fungal infection of plants, including some infections of major economic interest.<sup>120</sup> For example infection of bean and cotton plants by the soil fungi *Rhizoctonia solani* and *Sclerotium rolfsii* was effectively reduced by the presence of the *E. coli* ChiA-overproducing strain. In transgenic tobacco plants, expression of the ChiA gene improved resistance of the plants to fungal infection.<sup>121, 122</sup>

The bacterial type chitinase B from *S. marcescens* (*SmChiB*) is composed of a catalytic domain (residues 1–425), a linker (residues 426–450) and a small C-terminal (residues 451–499) domain referred as ChBD. This domain is formed by three antiparallel  $\beta$ -strands connected by long  $\beta/\alpha$ -loops. The catalytic domain consists of  $(\alpha/\beta)_8$  and an extra  $\beta/\alpha$  domain (residues 292–379), which provides a flexible loop (residues 310–325) near the active site. The active site is formed with a long, deep cleft lined by the exposed hydrophobic residues, as shown in Figure 5.<sup>123</sup>

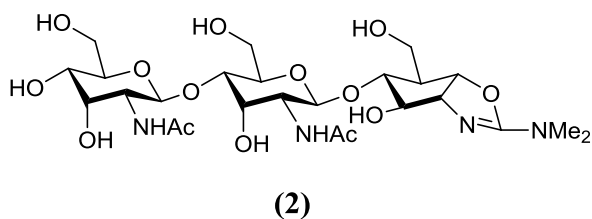


**Figure 5.** Sequence alignment of chitinase ChiB from *S. marcescens*, ChiA from *S. marcescens* and heveamine from *Hevea brasiliensis*. The catalytic regions of TIM barrel  $\beta$ -strands are indicated by  $\beta$ 1–8. Glu144 is part of the Family 18 DxxDxDxE motif. The following definitions of loops and domains are: the  $\alpha/\beta$ -domain (residues 295–373), the linker (residues 425–450) and the ChBD (residues 451–498), the porch loop (residues 14–27), the support loop (residues 233–262), the flexible loop (residues 315–325).<sup>123</sup>

### 3. Existence of natural product chitinase inhibitors

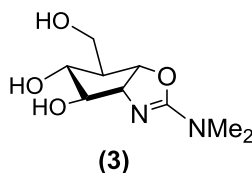
Most known chitinase inhibitors compete with the substrate by blocking its binding site, whether they mimic the reaction intermediate by their structure, or they mimic the key interactions with the conserved catalytic residues (Asp-175 and Glu-177 in *AfChiB1*).

#### 3.1. Allosamidin



**Figure 6.** Structure of allosamidin.

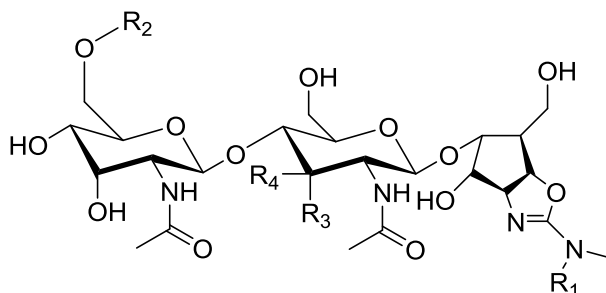
The pseudotrisaccharide allosamidin (Figure 6) is the most extensively studied chitinase inhibitor and inhibits many Family 18 chitinases in the nanomolar-micromolar range. Allosamidin is composed of two *N*-acetylhexosamine residues with an unusual *D-allo*-configuration and a novel aminocyclitol aglycone, named allosamizoline, joined by two  $\beta$ -(1,4)-glycosidic bonds. It was first isolated from the mycelium of *Streptomyces sp.*<sup>122</sup> The pseudotrisaccharide has been used to investigate the physiological role of chitinases involved in a variety of organisms. For example, potential activity of allosamidin as a chitinase inhibitor is shown by inhibition of cell separation in fungi,<sup>56</sup> toxicity towards insect larvae,<sup>79, 124</sup> blocking of malaria parasite penetration into the mosquito midgut<sup>125</sup> and easing of lung inflammation in a mouse model of asthma.<sup>109</sup> It binds to human chitotriosidase (HCHT) and *Coccidioides immitis* chitinase (*CiChi*) with  $K_i$  values of 40 nM and 230 nM respectively.<sup>47</sup> Allosamidin binds to different chitinases in a similar way, with the two *N*-acetylallosamine sugars occupying the -3 and -2 subsites in an identical fashion to the corresponding *N*-acetylglucosamine substrate units. The allosamizoline unit binds in the -1 enzyme subsite as an exact mimic of the oxazolinium ion reaction intermediate, making similar hydrogen bonds from the allosamizoline nitrogen and oxygen to the side chains of Asp-175 and Tyr-245, respectively.<sup>40, 52, 126</sup> Moreover, Asp-175 is in the “up conformation” forming an additional hydrogen bond with Glu-177, similar to that observed for the reaction intermediate.



**Figure 7.** Structure of allosamizoline.

Although the aglycone (3) itself is not an inhibitor, numerous efforts have been directed toward its synthesis, plus that of the carbohydrate core, in order to supply synthetic (2). The total

synthesis of (**2**) has been reported<sup>127</sup> and the preparation of the aglycone is typically long and complicated (*e.g.* 13 steps from D-glucosamine).<sup>128</sup>



**Figure 8.** Allosamidin and its derivatives.

**Table 1:** Structure of allosamidin and its derivatives.

	R <sub>1</sub>	R <sub>2</sub>	R <sub>3</sub>	R <sub>4</sub>
Allosamidin (ALLO) ( <b>2</b> )	CH <sub>3</sub>	H	OH	H
Demethylallosamidin (DEME) ( <b>2a</b> )	H	H	OH	H
Methylallosamidin (METH) ( <b>2b</b> )	CH <sub>3</sub>	CH <sub>3</sub>	OH	H
Glucoallosamidin B (GLCB) ( <b>2c</b> )	H	CH <sub>3</sub>	H	OH

A number of naturally occurring analogues of (**2**) are also known, in which the aglycone is *N*-demethylated or the sugar units modified, as seen Table 1.<sup>126</sup>

Chitinases from several yeasts and also human chitinases were efficiently inhibited by these allosamidins. The level of inhibition was dependent on the precise inhibitor structure, as shown in Table 2.<sup>126</sup>

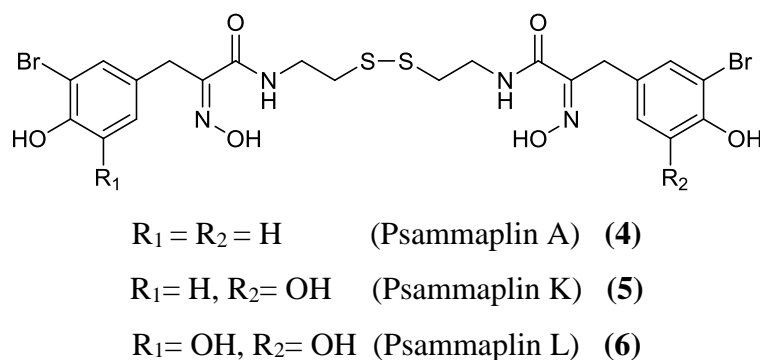
**Table 2.** IC<sub>50</sub> values of allosamidin derivatives against chitinases from different species.

	<i>S. cerevisiae</i>	<i>C. albicans</i>	<i>T. harzianum</i>	<i>B. mori</i>	Human
ALLO ( <b>2</b> )	55000	10000	1300	48	40
DEME ( <b>2a</b> )	480	1100	1300	81	1.9
METH ( <b>2b</b> )	60000	14000	1900	65	2.6
GLCB ( <b>2c</b> )	810	1300	2600	65	8.0

The chitinases from *S. cerevisiae* and *C. albicans* showed a 10-100-fold drop in IC<sub>50</sub> for DEME (**2a**) compared with ALLO (**2**), whereas chitinases from *Trichoderma harzianum* and *Bombyx mori* did not show this effect. In addition, HCHT (Human chitinase) and the chitinase from *T. harzianum* and *B. mori* bind ALLO (**2**) 10-100-fold better than the fungal chitinase from *S. cerevisiae* and *C. albicans*. These different affinities for the substrate are related to the construction of the active site groove. *S. cerevisiae* and *C. albicans* chitinases are similar to the relatively small plant chitinase hevamine, which lacks the extra  $\alpha/\beta$  domain.<sup>69</sup> By contrast, HTC contains the extra  $\alpha/\beta$  domain and two conserved hydrophobic residues Met-210 and Met-356 which interact with the methyl groups of the allosamizoline.<sup>126</sup>

Since the availability of (**2**) is limited and also the scope for design of novel allosamidin-derived inhibitors, this has prompted the search for other inhibitor molecules, both natural and synthetic.

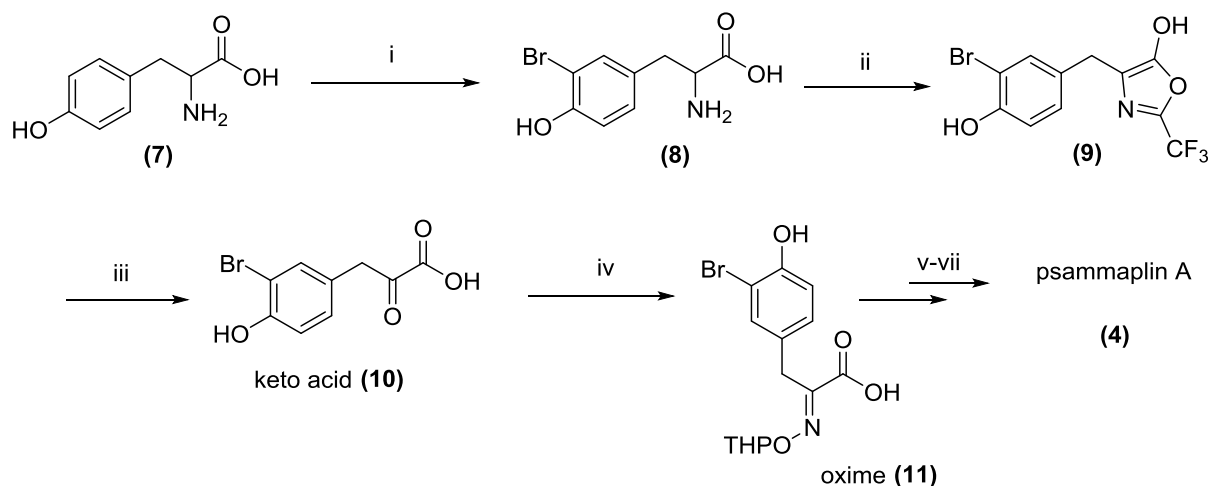
### 3.2. Psammaplins



**Figure 9.** Structures of Psammaplins.

Psammaplin A (**4**) is a member of a family of compounds with antibacterial and antitumour properties that are derived biosynthetically from bromotyrosine and cysteine. It was originally extracted from the sponge *Psammaplysilla purpurea*<sup>129</sup> and has biological properties against a *Bacillus* sp. chitinase with an IC<sub>50</sub> of 68  $\mu$ M and *Serratia marcescens* chitinase B (*SmChiB*) with a  $K_i$  of 148  $\mu$ M and an IC<sub>50</sub> of 100  $\mu$ M.<sup>47</sup> The structure of *SmChiB* in complex with (**4**) shows undefined electron density in the active site consistent with conformational flexibility and disordered binding of the inhibitor. Asp-175 is partially in the “up conformation” pointing towards Glu-177 indicating partial binding in the -1 subsite of the enzyme. The precise binding mode of this inhibitor with Family 18 chitinases has thus so far not been established.





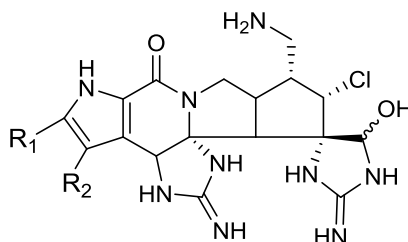
**Scheme 3.** Synthesis of Psammaplin A.<sup>130</sup>

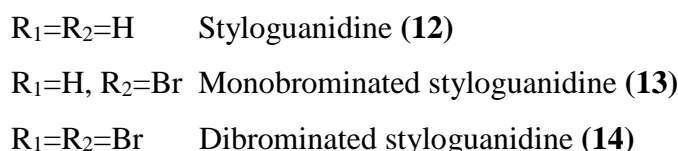
Reagents and conditions: (i) KBrO<sub>3</sub>, KBr, HCl, MeOH; (ii) TFAA, reflux; (iii) aq. TFA; (iv) THPONH<sub>2</sub>; (v) NHS, EDC, 1,4-dioxane; (vi) Et<sub>3</sub>N, cystamine, 1,4-dioxane/MeOH; (vii) HCl, CH<sub>2</sub>Cl<sub>2</sub>/MeOH.

Nicolau *et al.*<sup>130</sup> reported the synthesis of psammaplin A (**4**) starting from the commercially available amino acid L-tyrosine (**7**), which was monobrominated, then was converted to trifluoromethyloxazolone (**9**) by treatment with trifluoroacetic acid anhydride and subsequently hydrolyzed to give an  $\alpha$ -keto acid (**10**). To complete the synthesis, the  $\alpha$ -oxime (**11**) was formed by condensation of (**10**) with *O*-(tetrahydro-2H-pyran-2-yl)hydroxylamine, followed by the symmetric amide coupling of the  $\alpha$ -oxime with cystamine and cleavage of the THP group to give (**4**), as seen in Scheme 3.

Hong *et al.*<sup>131</sup> have subsequently reported an alternative approach to the key protected oxime derivative shown in Scheme 3 above, starting from a non-amino acid precursor. This potentially provides easier access to diverse psammaplin analogues.

### 3.3. Styloguanidines





**Figure 10.** Styloguanidine structures.

The styloguanidines (Figure 10) are a small group of natural products that possess a unique hexacyclic bis-guanidine structure. Styloguanidines and brominated analogues were isolated by Kato *et al.*<sup>132</sup> from the sponge *Stylotella aurantium*. They reported that styloguanidines showed activity towards a bacterial chitinase from *Schwanella sp.* and also inhibited the moulting of cyprid larvae of barnacles.

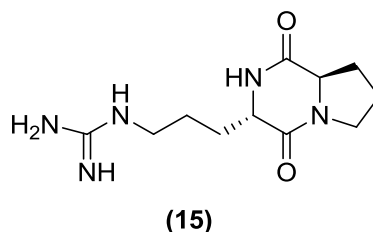
Chang *et al.*<sup>133</sup> have recently reported the synthesis of styloguanidines and related compounds. Lanman *et al.*<sup>134</sup> first reported the synthesis of the tetracyclic core of styloguanidines and several other advanced intermediates have also been reported by this group.<sup>134</sup> Other synthetic efforts towards the styloguanidines and related bis-guanidine natural products have also been reported by Hoffmann and Lindel.<sup>135</sup>

### 3.4. Cyclic Dipeptides

Although carbohydrate oligomers and their derivatives are good candidates for the design of glycosyl hydrolase inhibitors, they are often difficult to synthesize and too large to cross cell membranes. An alternative class of compounds which are readily available for the construction of chitinase inhibitors are peptides. Peptides have been used to mimic carbohydrate-protein interactions and bind to enzymes and lectins.<sup>136, 137</sup> While carbohydrates are critical molecules in the interaction between microbes and the host cell, they are not readily studied molecularly using genetic vectors and are poorly immunogenic. Therefore, the use of peptides (easy to produce, characterize and manipulate) as substitutes for natural polysaccharides has been developed. For example, in MethA tumour, a peptide mimetic of *O*- $\beta$ -linked *N*-acetylglucosamine present on the tumour surface-expressed glycoproteins stimulates the regression of the tumour *via* the activation of specific antitumour cellular responses.<sup>138</sup>

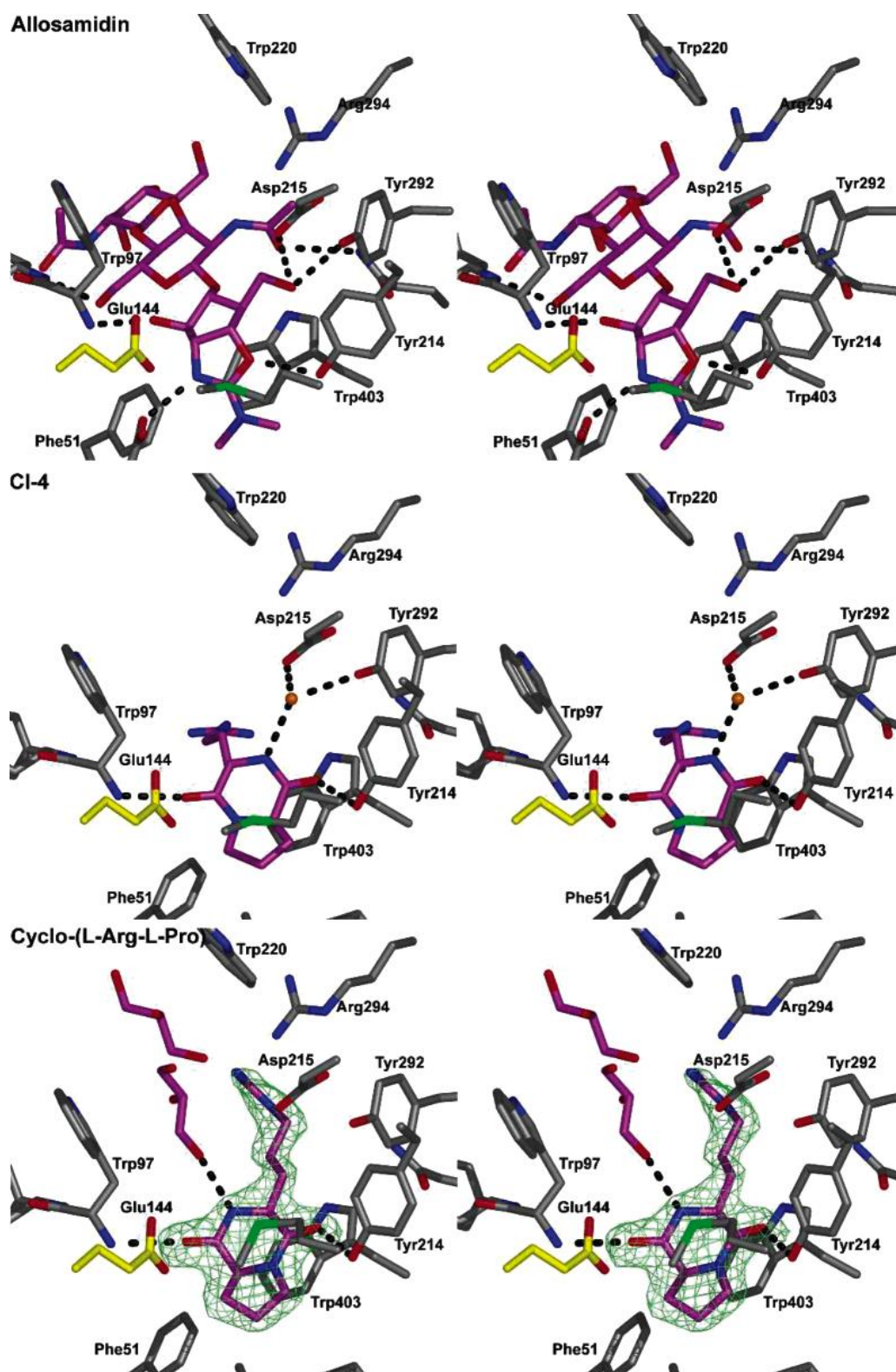
CI-4, argifin and argadin (see section 3.5) are all peptide-based inhibitors that mimic chitinase-carbohydrate interactions, both in terms of hydrogen bonds and stacking interactions.

### 3.4.1. CI-4, cyclo(L-Arg-D-Pro)



**Figure 11.** Structure of CI-4.

The cyclic peptide, cyclo (L-Arg-D-Pro) named CI-4 (Figure 11), produced by the marine bacterium *Pseudomonas* sp. IZ208, was isolated and identified as a chitinase inhibitor by Izumida *et al.*<sup>139</sup> CI-4 (**15**) and two stereoisomers were evaluated against *SmChiB*, with cyclo (L-Arg-D-Pro) showing a  $K_i$  of 0.65 mM and an  $IC_{50}$  of 1.2 mM, whereas an  $IC_{50}$  value of 6.3 mM was reported for the (D-L) isomer. Compound (**15**) inhibits separation of daughter cells in cultures of *Saccharomyces cerevisiae* and prevents the progression of the human pathogenic fungus *Candida albicans* from yeast to the infective filamentous form.<sup>140</sup>



**Figure 12.** Comparison of X-ray structure of *SmChiB* with allosamidin, CI-4 and cyclo-(L-Arg-L-Pro).<sup>141</sup>

Amino acids bordering the active site are shown as stick models and potential hydrogen bonds between the ligands (in the -1 subsite) and protein (including water- or glycerol-mediated) are represented as black dashed lines. The carbon atoms of the catalytic residue (Glu144) are yellow and those of the ligands are purple. The figure was made using PYMOL software (DeLano, W. L. The PyMOL MolecularGraphicsSystem (2002) <http://www.pymol.org>).

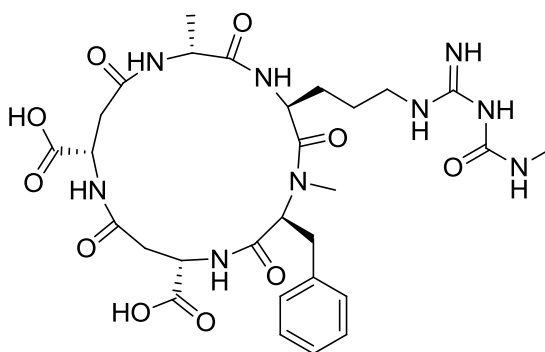
The structure of CI-4 mimics the intermediate formed in the reaction mechanism of all Family 18 chitinases - the five-membered oxazoline ring fused to a pyranose ring. The structure of *SmChiB* in complex with CI-4 (Figure 12) shows that it mimics the reaction intermediate and compared to allosamidin, CI-4 also makes the same interactions with the chitinase.<sup>141</sup> The six-membered ring of the CI-4 dipeptide backbone overlaps with the pyranose ring of the reaction intermediate. The proline ring occupies the same hydrophobic pocket as the oxazoline ring in the intermediate. The arginine side chain then occupies the same space as the *N*-acetylglucosamine in the -2 subsite. Hydrogen bonds are formed with Glu-144 (the catalytic acid of *SmChiB*), Trp-97, Tyr-214 and Asp-215 (water-mediated). Hydrophobic interactions are made with Ala-184, Met-212 and most notably the completely conserved Trp-408.

Compound (**15**) is a relatively weak inhibitor against *Serratia marcescens* chitinase with an  $IC_{50} = 1.2$  mM but because of its easy synthesis, it is a potential scaffold for derivatization.<sup>140</sup> The crystal structure of this compound in complex with *SmChiB* shows the Arg side chain does not make any significant interactions with the protein. This suggests that it could potentially be replaced with other groups that could stack against the hydrophobic amino acids present in the catalytic groove of the protein. Interestingly, the crystal structure of cyclo(L-Arg-D-Pro) showed that the orientation of the inhibitor in the active site depends on the stereochemical configuration of Pro. By changing the conformation, cyclo(L-Arg-L-Pro) is rotated 180° and subsequently the Arg side chain is pointed towards the + subsites, rather than the – subsites as in the (**15**)-ChiB complex.

### 3.5. Cyclopentapeptides

Cyclopentapeptide derivatives have been found to effectively inhibit the activity of chitinases because of their capacity to interact with the catalytic residues of these glycosyl hydrolases.

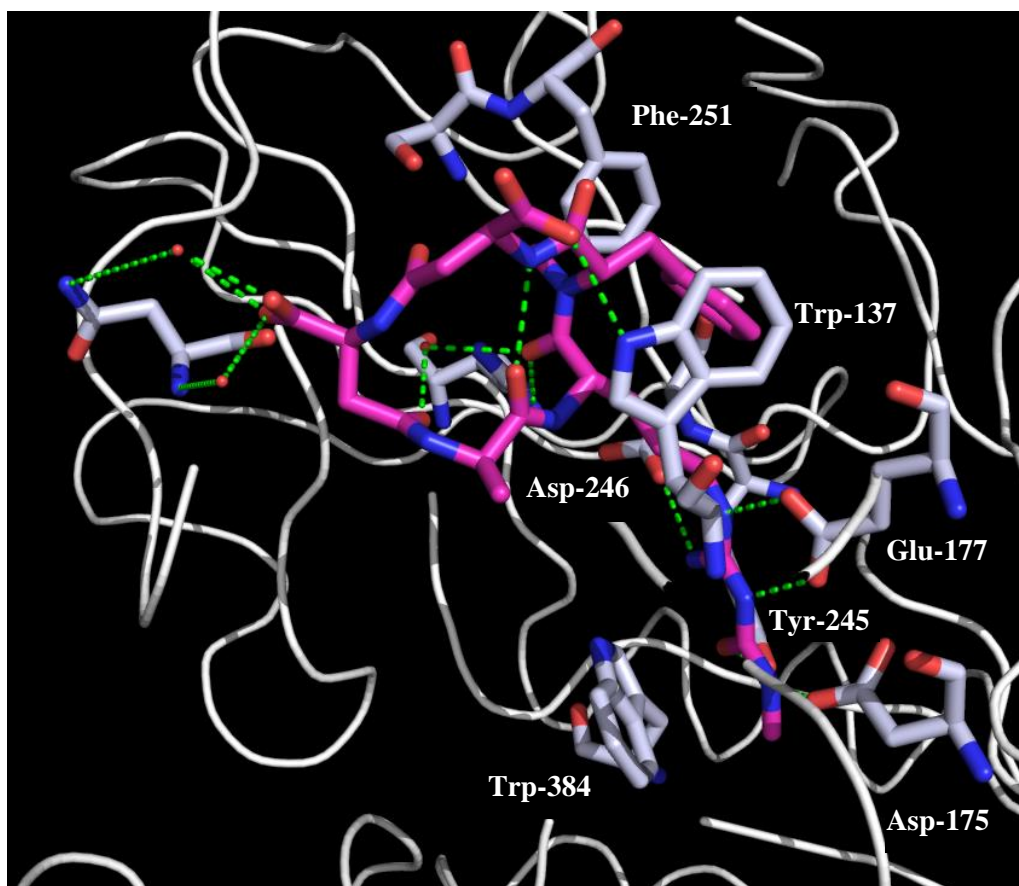
### 3.5.1. Argifin



(16)

**Figure 13.** Structure of argifin.

The cyclopentapeptide argifin (**16**) (Figure 13) was isolated from the fungal *Gliocladium* sp. FTD-0668<sup>142</sup> and was the first non-sugar molecule to show chitinase inhibition in the sub-micromolar range. Its structure consists of an *N*-methylcarbamoyl-derivatised L-Arg (Arg(MC)) an *N*-methyl-L-Phe residue (MePhe), two  $\beta$ -linked Asp residues and a D-Ala residue. The unusual acylated Arg residue plays a key role in the binding of (**16**) to Family 18 chitinases. IC<sub>50</sub> values of 3.7, 1.1 and 4.5  $\mu$ M were obtained against *Lucilia cuprina* (blowfly) chitinase, AfChiB1 and HCHT, respectively.<sup>117</sup>

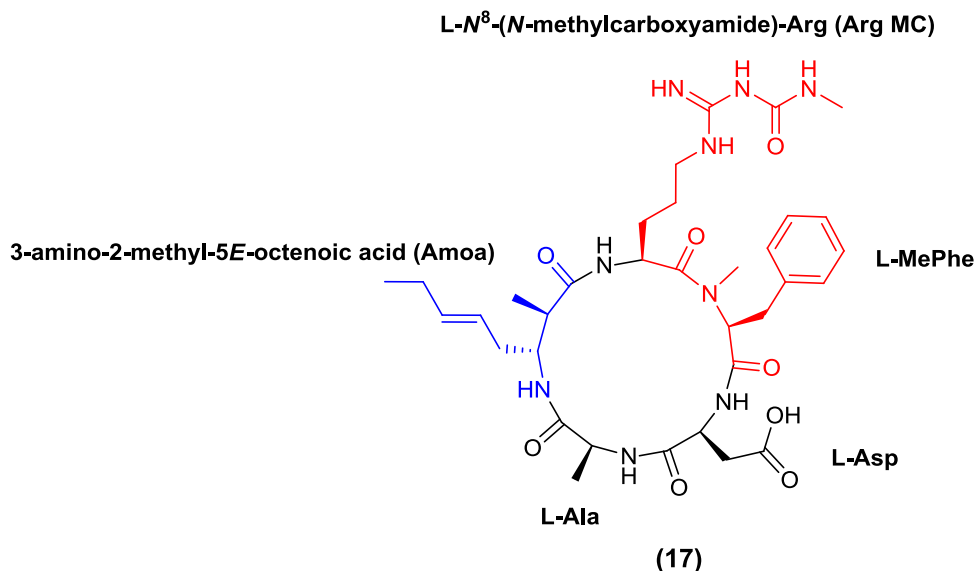


**Figure 14.** X-ray crystal structure of Argifin-AfChiB1 complex, with key active site residues indicated. (Image courtesy of Prof. D. M. F. van Aalten, University of Dundee).

Compound (**16**) binds to subsites -1, +1 and +2, interacting with residues Trp-384, Trp-137 and Phe-251 that are conserved hydrophobic residues in the Family 18 chitinases AfChiB1 (see Figure 14). The *N*-methylcarbamoyl moiety in the -1 subsite interacts with the side chain of Tyr-48 and Trp-384; its nitrogen hydrogen-bonds to the carboxyl groups of Asp-175 and Glu-177, whereas its oxygen hydrogen bonds to the hydroxy group of Tyr-245. The Arg residue also forms a salt-bridge-like interaction with the catalytic acid Glu-177. The *N*-methylcarbamoyl group, which resembles the *N*-acetyl group of the distorted -1 sugar in the SmChiB-GlcNA<sub>5</sub> structure, retains the hydrogen bonds to Asp-175 and Tyr-245. Likewise, one of the hydrogen bonds to the catalytic acid is preserved, while Asp-175 is retained in the “up conformation” pointing towards the catalytic acid.<sup>47</sup> Linear peptides containing an Arg(MC) residue and *N*-methylguanyllurea itself are also chitinase inhibitors.<sup>118, 143</sup> Andersen *et al.*<sup>143</sup> discovered through X-ray analysis that the ability of the guanyllurea moiety to penetrate fully into the active site pocket of the chitinases is strongly correlated with the inhibition of chitinase

activity. The synthesis of (**16**) by a combination of solution and solid-phase methods<sup>144, 145</sup> and all solid-phase methods has been reported.<sup>146, 147</sup>

### 3.5.2. Banyasin A



**Figure 15.** Banyasin A.

Banyasin A (**17**) (Figure 15) is a cyclopentapeptide isolated from the water bloom of the cyanobacterium *Nostoc sp.* (IL-235).<sup>148</sup> As for (**16**), the structure of (**17**) consists of an L-(N-methylcarbamoyl)-Arg (Arg-MC) residue, a L-MePhe residue, L-Asp and L-Ala residues and a novel amino acid, 3-amino-2-methyl-5*E*-octenoic acid (Amaa). The stereochemistry configuration at C2 and C3 in Amaa remain unresolved. The Arg (MC) unit contained in banyasin A is the essential feature that allows argifin to make key interactions with the enzyme AfChiB. Therefore it is of considerable interest to synthesize (**17**) and evaluate its potential activity against Family 18 chitinases.



### 3.5.3. Argadin

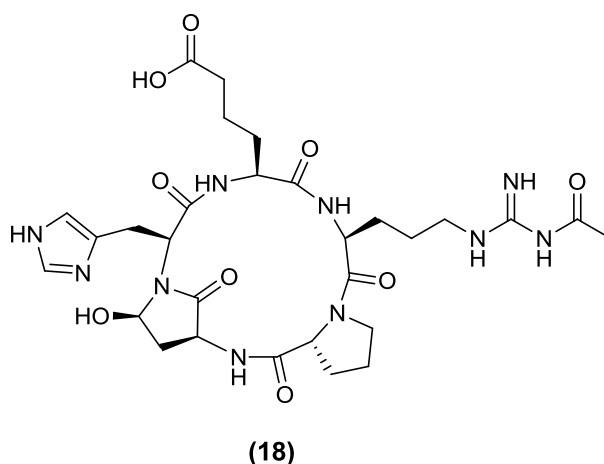


Figure 16. Argadin structure.

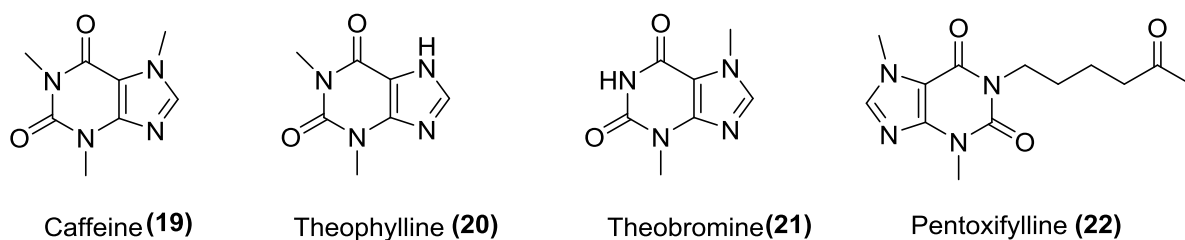
The cyclopentapeptide argadin (**18**) was isolated from a fungal strain *Clonostachys sp.* FO-7314<sup>149</sup> and is composed of an acetylated L-Arg residue, a D-Pro residue, a backbone cyclised L-Asp- $\beta$ -semialdehyde, an L-His residue and an L-aminoadipic acid residue. Its inhibitory potency against typical bacterial chitinases is greater compared to argifin (**16**) with IC<sub>50</sub> values of 150 nM against *Lucilia cuprina* chitinase,<sup>150</sup> 0.5  $\mu$ M against AfChiB1<sup>117</sup> and 13 nM against HCHT.<sup>117</sup> Compound (**18**) has also shown to arrest the moulting of cockroach larvae upon injection into the ventral abdominal part. The hemiaminal unit and the D-Pro residue restrict the rotation involving the main chain thus possibly contributing to a more rigid structure.

Dixon *et al.*<sup>144</sup> reported the synthesis of (**18**), with the peptide assembly being carried out by solid-phase synthesis, starting from the histidine residue. A homoserine residue was incorporated, which then was oxidised in solution to give an hemiaminal derivative, followed by the introduction of the derivatised Arg side chain. Hirose *et al.*<sup>151</sup> also achieved the synthesis starting from an L-Asp semialdehyde, which was linked directly to an acetal-type resin.

### 3.6. Methylxanthines

Methylxanthines (Figure 17) have been found in plants and include the well-known compounds caffeine (1,3,7-trimethylxanthine) (**19**), theophylline (1,3-dimethylxanthine) (**20**) and theobromine (3,7-dimethylxanthine) (**21**). These are purine alkaloids and are produced from

coffee (*Coffea arabica*), tea (*Camelia sinensis*), mate (*Ilex paraguariensis*), guarana (*Paullinia cupana*), cola (*Cola nitida*) and cacao (*Theobroma cacao*).



**Figure 17.** Structures of methylxanthines.

These compounds have a wide range of medical applications, including the treatment of asthma, bronchitis and chronic obstructive pulmonary disease and are also used as diuretics, cardiac stimulants and renal protective agents. Rao *et al.*<sup>152</sup> used a fluorescence-based assay of a commercially available library of 800 molecules screened at 100  $\mu$ M against the Family 18 chitinase, chitinase B1 from *A. fumigatus*. This identified the xanthine derivatives, theophylline (20) and caffeine (19) as competitive inhibitors of bacterial, fungal and human Family 18 chitinases.<sup>152</sup> They were shown to be competitive inhibitors of *AfChiB1* with (19) and (20) having  $IC_{50}$  values of 469  $\mu$ M and 1500  $\mu$ M, respectively. They were crystallographically determined to bind to the active site of *AfChiB1*, again mimicking the oxazolinium ion intermediate by hydrogen-bonding to the side chains of Asp-175 and Tyr-245, as well as the main chain of Tyr-137. The 1,3-dimethylxanthine substructures of these inhibitors also stack in the active site, making hydrophobic interactions with the conserved tryptophan residues in the active site of the protein.

## 4. Synthetic chitinase inhibitors

### 4.1. Bacterial-type chitinase inhibitors

In the high-throughput screen described above,<sup>149</sup> the most promising compounds identified were the natural methylxanthine, caffeine (19) and the synthetic derivative, pentoxifylline (22) is the most potent of the three methylxanthine inhibitors identified against *AfChiB1* with a  $K_i$  of 37  $\mu$ M. The *AfChiB1*-(22) complex reveals several unusual interactions which may explain the increase of magnitude in potency compared to caffeine. Strikingly Trp--137 rotates 93°

around (22), sandwiching the xanthine moiety with extensive  $\pi$ - $\pi$  stacking interactions. The rotation of Trp-137 excludes an ordered water molecule seen in the complexes with (20) and (19). Additionally the alkyl tail of (22) extends to the -3 subsite, replacing the second ordered xanthine seen in both the complexes of (19) and (20), stacking with Trp52 and hydrogen bonding two ordered water molecules.

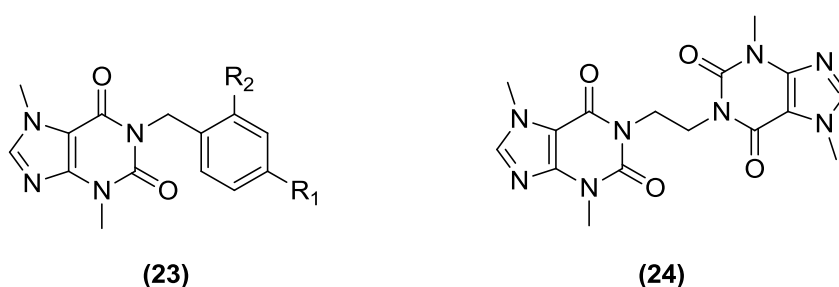
Compound (20) is the weakest of the three inhibitors with an  $IC_{50}$  1500  $\mu$ M.<sup>152</sup> The inhibitor binds in a position equivalent to the allosamidin allosamizoline unit. Allosamizoline is known to mimic the oxazolinium intermediate. The methylxanthine mimics this intermediate by forming hydrogen bonding with Asp-175, Tyr-245 and the backbone nitrogen of Trp-137, residues all conserved in Family 18 chitinases. In addition, the methylxanthine moiety makes favourable  $\pi$ - $\pi$  stacking interactions with Trp-387. Compared to (20), (19) contains an additional 7-methyl group, which appears to increase the inhibition 3-fold to an  $IC_{50}$  460  $\mu$ M. This results in the loss of the hydrogen bond for the xanthine-N7 with Asp-175 but also forces Asp-175 in to a down conformation compared to the up conformation observed in the *AfChiB1*-allosamidin. Similarly Glu-177 moves away to avoid steric clashes of the terminal carboxylate with the *N*-7 methyl. As well as the methylxanthine in the -1 subsite, an additional ordered inhibitor molecule is observed in the *AfChiB1*-(19) complex occupying a position equivalent to the -3 sugar observed in the *AfChiB1*-allosamidin complex. This additional methylxanthine molecule also makes stacking interactions with Trp-52, which is conserved in the other bacterial-type chitinases, such as human chitotriosidase, AMCase and chitinase A from *Serratia marcescens*.

The largest of the methylxanthine inhibitors, (22), only covers a small part of the *AfChiB1* active site, from the -1 to just beyond the -2 subsite. By comparison, other more potent chitinase inhibitors, such as allosamidin (2) (which covers the -1 to -3 subsites) and argifin/argadin (16)/(18) (which cover the -1 to +2 subsites) occupy further subsites. It should be noted that nearly all residues in the -1 subsite are identical in the “bacterial”- and “plant”-types of chitinases, suggesting that these inhibitors may also reduce activity of the latter class of enzymes. The simple xanthines thus have a rather poor affinity for chitinases, with a  $K_i$  of 37  $\mu$ M for (22) against fungal chitinase *AfChiB1*.<sup>152</sup> However, it was recognised that it should be possible to extend the methylxanthine scaffold to address additional available chitinase subsites and increase chitinase affinity and selectivity through synthetically accessible purine chemistry. Molecules of this nature may help elucidate the role that chitinases play in fungal

morphology, growth and virulence. Similarly, this approach may extend to the study of the precise functions of the mammalian chitinases, which share sequence homology with *AfChiB1*. In this regard, targets could be chitotriosidase and acidic mammalian chitinase, as well as several structurally related Family 18 chitinase-like lectins (chilectins), purported to be involved in carbohydrate recognition, such as HCgp-39, YM-1 and YM-2 that have been implicated in the pathophysiology of inflammation.

The three 1,3-dimethylxanthine derivatives, (**19**), (**20**) and (**22**), possess a common binding mode with Family 18 chitinases, whether making  $\pi$ - $\pi$  interactions, or mimicking the hydrogen bonds of the reaction intermediate in the active site of the enzyme. The xanthine moiety of the three compounds always binds in the same orientation as observed for the *AfChiB1* in the active site. Additionally (**22**) offers good ligand efficiency,  $\Delta G = -0.43 \text{ Kcal mol}^{-1} \text{ atom}^{-1}$ , suggesting that it should be an ideal starting point for further optimization.<sup>153</sup>

Schüttelkopf *et al.*<sup>153</sup> screened a database of 5.1 million commercially available compounds for molecules containing the 3-methylxanthine substructure, as this moiety makes the key interactions with the enzyme (*AfChiB1*). 50,193 candidate ligands were identified. The most promising hits were evaluated by docking with PRODRG/LIGTOR and twelve compounds were then selected for evaluation *in vitro*. Compounds (**23**) and (**24**), shown in Figure 18 showed significant inhibition, but for (**23**), its activity was worse than caffeine, so it was not considered for any further studies.



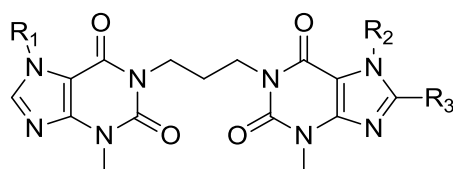
**Figure 18.** 3-Methylxanthine scaffolds as potential inhibitors of *AfChiB1*.

Compound (**24**) showed an  $IC_{50} = 4.8 \pm 1.4 \mu\text{M}$ , improving inhibition by 2 orders of magnitude compared to caffeine (**19**) and nearly 25- fold compared to pentoxifylline (**22**), the most potent xanthine-based chitinase inhibitor. “C<sub>2</sub>-dicaffeine” (**24**) is a competitive inhibitor of chitinase

with an extensive  $\pi$ - $\pi$  stacking binding mode. Defined electron density for (24) shows the “primary” caffeine moiety is sandwiched between Trp-137 and Trp-384, whereas the “second” stacks with Trp-52.<sup>153</sup>

Compound (24), C<sub>2</sub>-dicafeine or bisdionin B, inhibits several bacterial type Family 18 chitinases.<sup>153</sup> The complex of AfChiB1-(24) showed significant stacking interactions between the three tryptophan residues, Trp-52, Trp-137 and Trp-384 residues. This suggested that (24) can be used as ligand to test for inhibition of bacterial-type chitinases. Subsequently, three additional Family 18 chitinases were screened against (24), namely human macrophage chitotriosidase (HCHT), acid mammalian chitinase activity from mouse lung (mAMCase) and a bacterial-type enzyme from *S. marcescens* (SmChiB). The IC<sub>50</sub> values were 107  $\mu$ M, 90  $\mu$ M and 71  $\mu$ M, respectively. The substitutions of the amino acids within the catalytic site of each protein which do not interact directly with the xanthine units seem to affect more or less the activity of (24) against these enzymes. This suggested that dicafeine molecules could be used as scaffolds to design inhibitors of bacterial type chitinases in general and selectively target individual enzymes.<sup>153</sup>

In the crystal structure of the AfChiB1-(24) complex,<sup>153</sup> the active site Asp175 is in the down conformation into the catalytic core, like in other complexes with xanthine derivatives. Furthermore there are two major differences from the previously known xanthine binding mode and the docked conformation: first, (24) is shifted out of the active site by about 1.5 Å and second, both purine rings are flipped by 180° around their N-1 linker bond. This binding mode significantly improves  $\pi$ - $\pi$ -stacking between the secondary caffeine and Trp-52 compared with the docked orientation.<sup>153</sup>



R<sub>1</sub> = R<sub>2</sub> = CH<sub>3</sub>, R<sub>3</sub> = H Bisdionin C (25)

R<sub>1</sub> = CH<sub>3</sub>, R<sub>2</sub>, R<sub>3</sub> = H Bisdionin F (26)

R<sub>1</sub> = CH<sub>3</sub>, R<sub>3</sub> = CH<sub>3</sub>-CH<sub>2</sub> Bisdionin G (27)

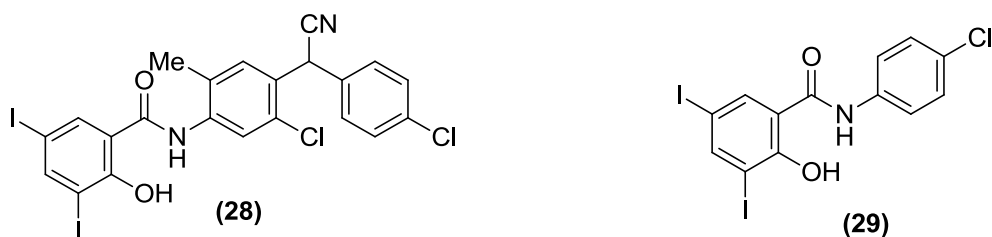
**Figure 19.** Structures of bisdionins.

C<sub>3</sub>-dicaaffeine also known as bisdionin C, (**25**) is also a drug-like molecule based on Lipinski's rule of five. Compound (**25**) is more active against *AfChiB1* compared to (**24**) due to its longer linker between the two xanthines. Bisdionins C (**25**) and F (**26**) showed inhibitory activity against *AfChiB1* and AMCase with IC<sub>50</sub> values of 0.20  $\mu$ M and 2.2  $\mu$ M respectively.<sup>154</sup>

Bisdionin G (**27**), a derivative of bisdionin F (**26**) carrying an ethyl substituent on the 8-position of the second xanthine moiety, has recently been identified as an effective ligand of chitinase-like protein YKL-39, with a  $K_i$  value of  $120 \pm 2$   $\mu$ M.<sup>155</sup>

#### 4.1.2. Closantel plus related derivatives

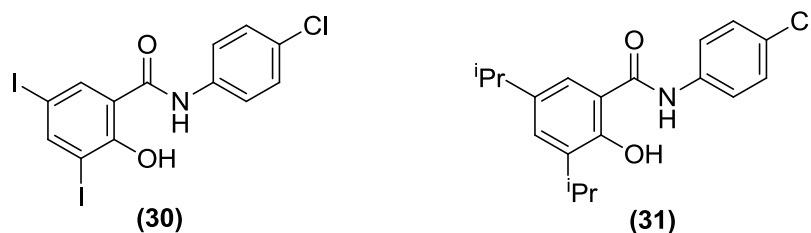
The neglected tropical disease, river blindness or *Onchocerciasis* is caused by the filarial parasitic nematode *Onchocerca volvulus*, which is transmitted to humans by blackfly. Its symptoms are the result of the death of the microfilarae in the skin and eyes. Recently, one chitinase from *O. volvulus*, *Ov*CHT1, has been identified and it is expressed in larvae and stored within cells of the oesophageal glands of the nematode. This larval chitinase has a role in parasite transmission, ecdysis and remodelling of cuticle. Consequently deregulation of this metabolism is a potential therapeutic target to develop inhibitors of *Ov*CHT1. Gloeckner *et al.*<sup>116</sup> used a fluorescence-based assay, to screen the Johns Hopkins Clinical Compounds Library (JHCCL), a commercially available library of 1514 known drugs, against *Ov*CHT1. They found that the veterinary anthelmintic drug closantel (**28**) is a chitinase inhibitor with an IC<sub>50</sub> 1.6  $\mu$ M. Additionally, closantel was also found to inhibit the L3 to L4 larval moult in *O. volvulus* in the same manner as had been observed with cysteine protease and transglutaminase inhibitors cultured with this parasitic species. Structure retrofragmentation of (**28**) led to compound (**29**), which was identified as a chitinase inhibitor with an IC<sub>50</sub> = 5.8  $\mu$ M, similar to (**28**).



**Figure 20.** Structure of closantel (**28**) and its derivative (**29**).

Because of the role of (**28**) as a protonophore and its chitinase inhibitor activity, this suggests a potential bi- or multimodal binding mechanism with *Ov*CHT1.

To investigate the molecular fragment responsible for the individual and or dual biochemical activities of (28), a series of compounds based on the closantel fragment (29) was screened for chitinase inhibition and protonophore activity. These studies revealed that (30) has a better chitinase inhibition with  $IC_{50} = 0.63 \mu M$  compared to (28) and has no protonophore activity. Additionally compound (31) showed no chitinase inhibition but was found to be a more potent ionophore than (28).

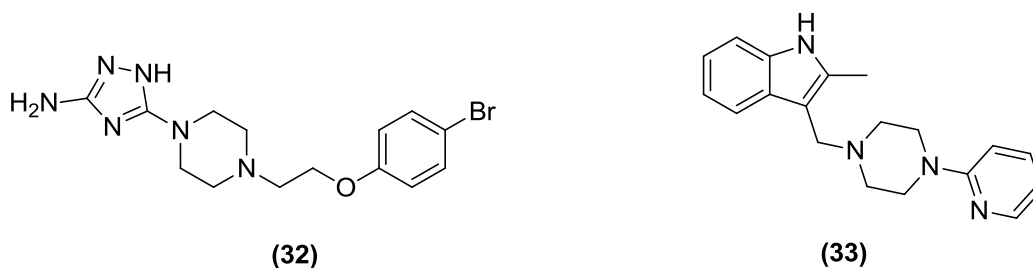


**Figure 21.** Structure of *Ov*CHT1 inhibitors.

#### 4.1.3. AMCase inhibitors from high throughput screening

The Acidic Mammalian Chitinase (AMCase), which is a member of the Family 18 chitinases, has been recently demonstrated to be implicated in the pathophysiology of asthma. Several natural products or novel synthetic derivatives have been shown to be inhibitors of AMCase.<sup>155</sup> To identify new small molecule inhibitors of AMCase, Cole *et al.*<sup>156</sup> applied a combination of high-throughput screening (HTS), fragment based screening (FBS) and virtual screening (VS) techniques.

The AMCase HTS assay based on hydrolysis of the fluorescent-based substrate, 4-methylumbelliferyl( $\beta$ -D-*N,N'*-diacetylchitobioside) was used to screen a library of 446,000 compounds at a concentration of  $10 \mu M$ .<sup>156</sup> The structures of the most promising hits from HTS are shown below in Figure 22.

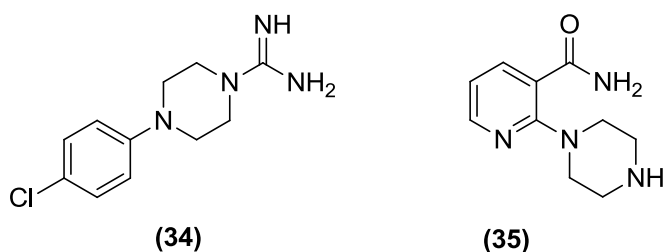


**Figure 22.** Structure of AMCase inhibitors.

The X-ray structure of these inhibitors in complex with AMCase revealed that **(32)** was a potent inhibitor.<sup>156</sup> Its structure was compared to the previous structure of AMCase with methylallosamidin (**2a**). The aminotriazole moiety of **(32)** was found to bind in the active site forming hydrogen bonds with catalytic residues Asp-138, Glu-140 and Tyr-212 and to stack against the conserved residues Trp-360 and Met-210. The rigid bromophenyl ring interacts with hydrophobic residues Tyr-237, Ala-295 and Ile-300.

The compound **(33)** binds to AMCase in the same manner as for the compound **(32)**, with the pyridine ring occupying the conserved residues Trp-360 and Met-210 and the piperazine moiety overlapping with Tyr-237, Ala-295 and Ile-300. The indole ring by extension makes hydrophobic interactions with Tyr-267, Ala-295, Ile-300 and Leu-364.

Cole *et al.*<sup>156</sup> screened other hits identified by FBS shown in Figure 23 that are weaker inhibitors than **(32)** and **(33)** but have lower molecular weights and clogP. This means that the ligand efficiency (LE) and ligand-lipophilic efficiency (LLE) of these compounds **(34)** and **(35)** are comparable, plus they are equally good (hits of  $IC_{50} < 0.1-100 \mu M$ ), so they can be used as scaffolds for further optimization.



**Figure 23.** Structures of AMCase inhibitors identified by FBS.

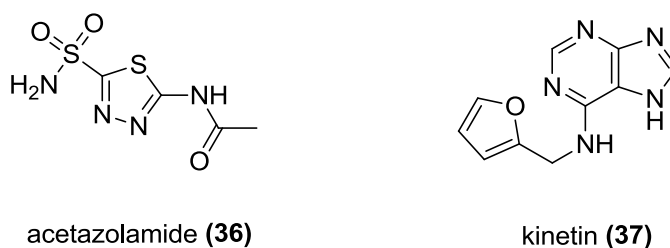


## 4.2. Plant chitinase inhibitors

Plant-type chitinases are involved in fungal cell-wall remodelling during cell division. Dereglulation of this process may result in a decrease of fungal viability as well as virulence, making inhibitors of plant-type chitinases potential tools as antifungal agents.

To date, mainly bacterial-type chitinase inhibitors have been reported and most of them are weak inhibitors against fungal plant-type Family 18 chitinases. The crystal structure of the plant-type fungal chitinase, *Saccharomyces cerevisiae* CTS1 (ScCTS1) was established by Hurtado-Guerrero *et al.*<sup>157</sup> This protein displays four domains: a signal peptide, a catalytic domain, a Ser/Thr-rich domain and high-affinity chitin-binding regions. The active site consists of six sugar-binding subsites, numbered from -4 (the nonreducing end) to +2 (the reducing end) with hydrolysis taking place on the glycosidic bond between the -1 and +1 subsites.

In order to design antifungal compounds and to understand the role of plant chitinases in cell wall morphogenesis, Hurtado-Guerrero *et al.*<sup>157</sup> screened ScCTS1 against the Prestwick chemical library of 880 drug-like molecules. From this, three significant hits were identified: 8-chlorotheophylline ( $K_i$  of 600  $\mu\text{M}$ ), acetazolamide (**36**) a well-known carbonic anhydrase inhibitor and kinetin (**37**) (a plant hormone). Both (**36**) and (**37**) were competitive inhibitors, with  $K_i$  values of 21 and 3.2  $\mu\text{M}$ , respectively.



**Figure 24.** Competitive inhibitors of ScCTS1.

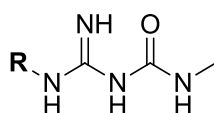
Allosamidin (**2**), a potent Family 18 chitinase of both plant and bacterial-type chitinases,<sup>122</sup> inhibits ScCTS1 with a  $K_i$  of 0.61  $\mu\text{M}$ <sup>157</sup> and hevamine with a  $K_i$  of 3.1  $\mu\text{M}$ <sup>158</sup> but it is a weak inhibitor for the plant-type chitinase A1 in *A. fumigatus* with an  $\text{IC}_{50}$  of 127  $\mu\text{M}$ .<sup>159</sup> Based on these studies, (**36**) and (**37**) were screened against *Af*ChiA1. The results showed that (**37**) failed to display any activity against *Af*ChiA1, even at higher concentrations. Compound (**36**) on the other hand, inhibited *Af*ChiA1 with an  $\text{IC}_{50}$  of 164  $\mu\text{M}$ .<sup>159</sup>

The X-ray structure of *Af*ChiA1-(**36**) shows that the thiadiazole ring stacks with the conserved Trp-312, while its ring nitrogens form hydrogen bonds with Ala-124 and Tyr-125. The acetamido moiety enters and fills the shallow active site pocket of *Af*ChiA1, by forming hydrogen bonds with Tyr-238, Gln-230, Met-310, Ala-205, Tyr-34 and Asp-172. The sulfonamide group makes a poor hydrogen bond with Trp-312.<sup>159</sup>

These investigations showed that (**36**) is a weak inhibitor of both *Sc*CTS1 and *Af*ChiA but, because of its synthetically accessibility and high ligand efficiency ( $-0.40 \text{ kcal mol}^{-1} \text{ atom}^{-1}$ ), it is an interesting lead for future inhibitor development.<sup>159</sup>

#### 4.2.1. Guanylurea and derivatives

The structure of argifin (**16**) in complex with *Af*ChiB1 has been used to design peptide fragments that contain the dimethylguanylurea unit, which penetrate fully into the active site groove.<sup>117, 160</sup> The potency of the argifin peptide fragments can be ranked tetrapeptides > tripeptide > mono peptide > dimethylguanylurea. Crystallography studies of these peptide-protein complexes and enzymology showed that these argifin fragments conserved their mode of binding and are still competitive inhibitors of Family 18 chitinases but with better ligand efficiency than (**16**), with BEI values 11.7 and 10.2 for (**16**) and the tetrapeptide, Ac-Arg(MC)-MePhe- $\beta$ -Asp-OH, respectively. Surprisingly, the simple dimethylguanylurea derivative (**38**) shown in Figure 25 and Table 3 forms most of the key interactions with *Af*ChiB1 and represents an unusually efficient binder and an attractive scaffold for structure-based optimization. In the (**38**)-*Af*ChiB1 complex, the fragment is well ordered in the same manner as the Arg(MC) side chain of (**16**) and it is able to make five hydrogen bonds within the active site.<sup>143</sup>



**Figure 25.** Structure of alkylguanylureas (see Table 3).

As the catalytic groove of Family 18 chitinases is strongly conserved,<sup>41-44</sup> it was speculated that a guanylurea derivative could also inhibit the plant-type Family 18 enzymes and that it should be possible to design specific inhibitors for this class of chitinases based on this simple molecular fragment.<sup>143</sup>

Remarkably, compound (**38**) was found to inhibit the plant-type enzyme *AfChiA1* with an  $IC_{50}$  of 79  $\mu$ M, confirming that effective inhibitors for this class of chitinases can be generated based on the guanylurea structure. A small series of simple “argifin-derived” fragments were synthesized and tested against *AfChiA1* as seen in Table 3. Note that three of the derivatives, (**38**), (**40**) and (**41**) are significantly more active than the best inhibitor of plant-type enzymes, allosamidin (**2**) with an  $IC_{50}$  of 127  $\mu$ M against *AfChiA1*.<sup>118</sup>

**Table 3.** Alkylguanylurea biological activities against *AfChiA1*

Compound	R	<i>AfChiA1</i> $IC_{50}$ ( $\mu$ M)
<b>38</b>	CH <sub>3</sub>	79 $\pm$ 8
<b>39</b>	CH <sub>3</sub> CH <sub>2</sub>	310 $\pm$ 7
<b>40</b>	CH <sub>3</sub> (CH <sub>2</sub> ) <sub>3</sub>	38 $\pm$ 7
<b>41</b>	Cyclo hexane	42 $\pm$ 1.3
<b>42</b>	PhCH <sub>2</sub>	299 $\pm$ 4

The exposed site of *AfChiB1* contains a larger  $\beta/\alpha$ -loops plus the incorporation of an entire  $\alpha/\beta$  domain and this is lined by the several solvent-exposed aromatic residues, including Trp 137. Whereas in the *AfChiA1* the active site is defined by very short  $\beta/\alpha$ -loops, the extra  $\alpha/\beta$  domain is lacking.

Table 3 shows that the alkylguanylureas (**38-42**) are efficiently accommodated in the shallow active site of the *AfChiA1*.

It is these differences in construction of the active site that explain the different affinities for argifin shown by *AfChiA1* and *AfChiB1*.<sup>118</sup>

### 4.3. Objectives of the project:

This project is focused on the synthesis and development of peptide-based chitinase inhibitors. Our specific objectives were to:

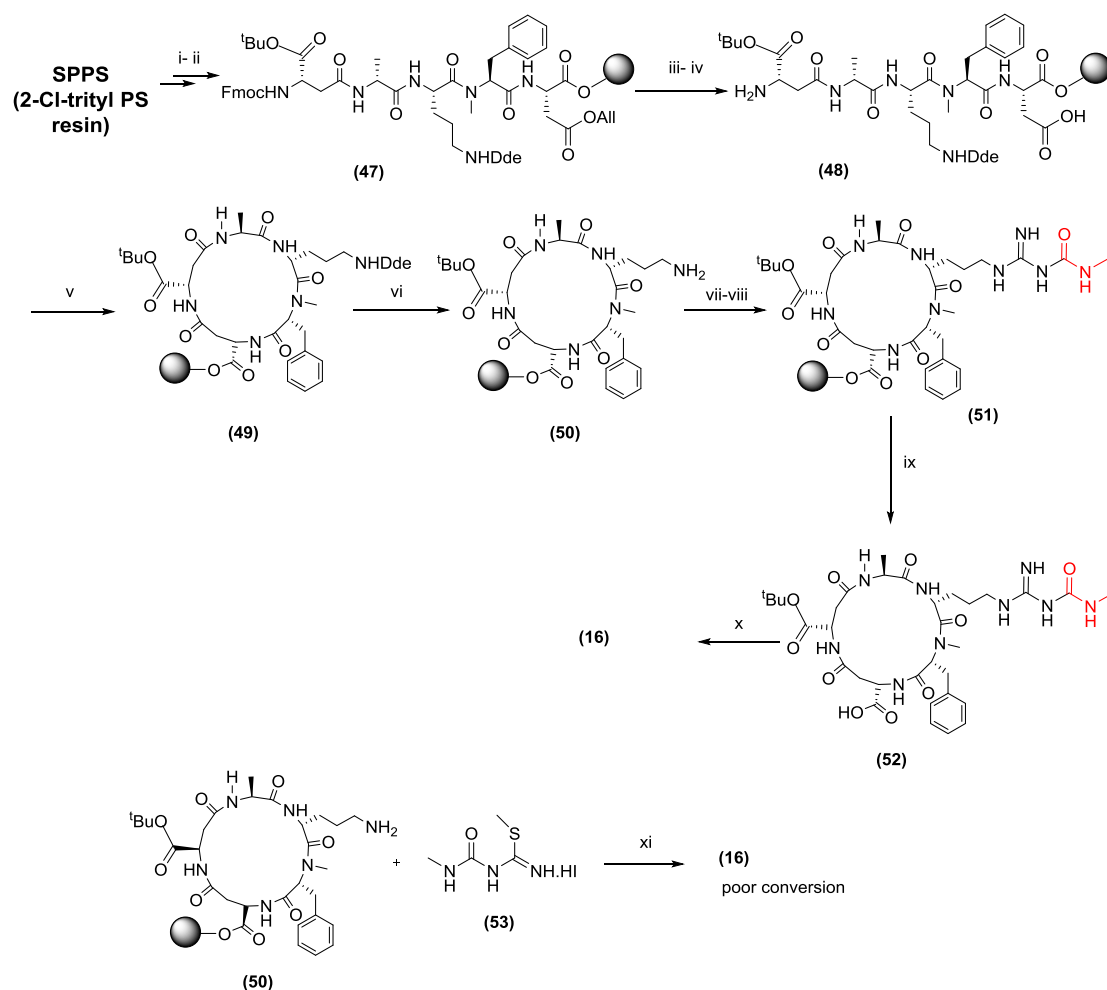
- Develop an improved synthesis of the cyclic pentapeptide argifin. Specifically, herein aim to develop a synthesis which is suitable for larger-scale preparation of the natural product and avoids side reactions that may occur in existing syntheses.
- Investigate the synthesis of new reagents for the direct conversion of amines to the key guanylurea unit of argifin and related peptides.
- Develop an efficient synthetic route to Amoa (3-amino-2-methyl-5-*E*-octenoic acid), the novel amino acid component of the cyclic peptide banyasin A. It was aimed to develop a synthesis that is suitable for the preparation of all four diastereoisomers of Amoa for incorporation into peptide synthesis.
- Synthesize and evaluate a series of analogues of the cyclic dipeptide inhibitor CI-4.



critical in the assembly of the linear precursor. The use of *tert*-butyl protection for the non-resin-linked Asp residue rather than benzyl ester protection reduced base-induced aspartimide formation<sup>162</sup> during the Fmoc deprotection. The cyclisation of the linear peptide was then performed in dilute CH<sub>2</sub>Cl<sub>2</sub> solution using PyBOP activation and DIPEA as base.

The cyclisation was effective as the choice of the linear precursor sequence maximises the possibility of favourable precyclised conformations, by having the MePhe and D-Ala residues in the second and fourth positions respectively. To complete the synthesis, the removal of *tert*-butyl and Pmc protecting groups was required. These acid-labile protecting groups were cleaved using a mixture of TFA/thioanisole/CH<sub>2</sub>Cl<sub>2</sub>/water (16:2:1:1, v/v/v). However the deprotection proved to be problematic leading to several products, including aspartimide formation.<sup>162</sup> Preparative HPLC was required in order to isolate the fully deprotected cyclic pentapeptide (**45**) in 28% yield. Finally, the derivatisation of the Arg residue was carried out using succinimidyl *N*-methylcarbamate in the presence of DBU as base. Mono-acylation gave the required Arg(MC) moiety. HPLC was again required in order to isolate the desired compound argifin (**16**), in 17% overall yield, based on the loading of the first amino acid in the solid-phase synthesis.

In summary, in the first synthesis of argifin,<sup>161</sup> removal of acid-labile Asp and Arg side chain protecting groups proved to be problematic, leading to aspartimide formation upon side chain deprotection and, as a consequence, two HPLC purifications were required during the synthesis. This is both time-consuming and reduces the overall yield of the cyclic peptide that can be obtained. In order to improve the efficiency of production of argifin and develop SAR on analogues of the cyclopentapeptide, another synthesis was therefore developed by Dixon *et al.*<sup>146</sup> as outlined in Scheme 5.



**Reagents and conditions:** (i) 2-chlorotrityl chloride polystyrene resin, DIPEA, CH<sub>2</sub>Cl<sub>2</sub>, 60 min; (ii) Fmoc SPPS; (iii) Pd(Ph<sub>3</sub>P)<sub>4</sub>, PhSiH<sub>3</sub>, CH<sub>2</sub>Cl<sub>2</sub>, 3 x 20 min; (iv) piperidine/DMF (1:4, v/v), 4 x 3 min; (v) PyBOP, DIPEA, CH<sub>2</sub>Cl<sub>2</sub>, 2 x 60 min; (vi) N<sub>2</sub>H<sub>4</sub>/DMF (1:49, v/v), 2 x 15 min; (vii) 1*H*-pyrazole-1-carboxamide hydrochloride, DIPEA, DMF, 16 h; (viii) MeNHCO<sub>2</sub>Su, DBU, DMF, 2 h; (ix) TFA/CH<sub>2</sub>Cl<sub>2</sub> (1:99, v/v), 10 x 20 min; (x) 1 M aq. HCl, 60 °C, 90 min; (xi) DIPEA, DMF, 2 x 16 h.

**Scheme 5.** Dixon's on-resin synthesis of argifin.<sup>146</sup>

The alternative synthesis of argifin as outlined in Scheme 5 was carried out by all solid-phase chemistry.<sup>146</sup> The acid-labile 2-chlorotrityl chloride polystyrene resin was again chosen for Fmoc-solid phase synthesis. The linkage to the resin was achieved through the  $\alpha$ -carboxyl of one of the Asp residues in the linear peptide precursor, with allyl ester protection on the  $\beta$ -carboxyl of this residue. The Orn side chain was Dde-protected, as Dde is removable by hydrazinolysis but stable to both Fmoc and allyl ester deprotection conditions. The non-resin-linked Asp residue was again protected as a *tert*-butyl ester (on the  $\alpha$ -carboxyl) in order to eliminate aspartimide formation during assembly of the linear peptide precursor (48). The use

of 2-chlorotrityl resin also helped suppress aspartimide formation when the resin-bound cyclic peptide intermediate (**50**) was exposed to the basic conditions required for the final Arg acylation step (step (viii) in Scheme 5).

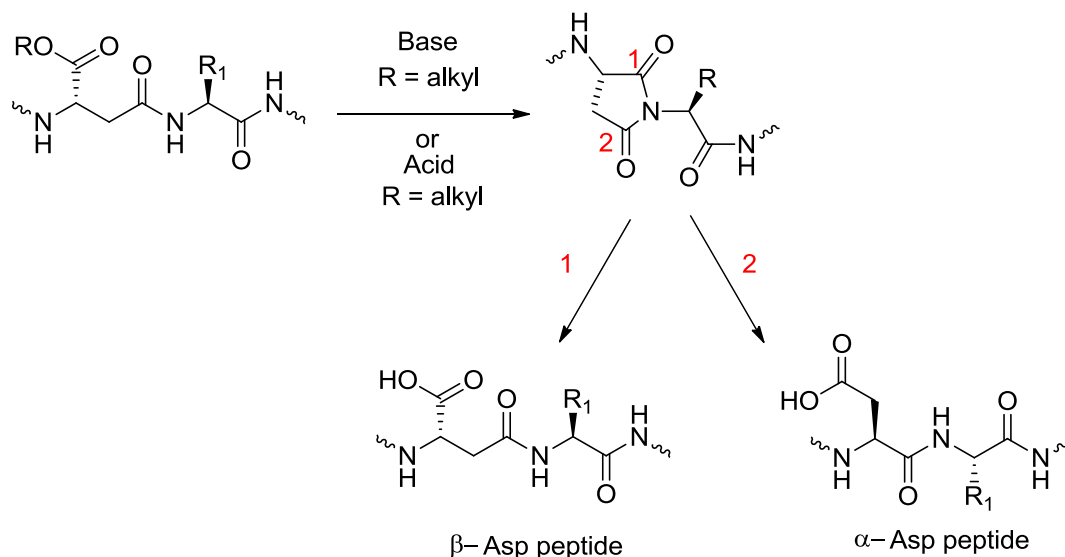
After removal of the Fmoc group on the *N*-terminal Asp residue and removal of the allyl ester protection at the C-terminal Asp, the cyclisation of the linear pentapeptide intermediate (**48**) was performed on resin in CH<sub>2</sub>Cl<sub>2</sub> solution using PyBOP activation and DIPEA as base. Cleavage of Dde from the resin-bound cyclic pentapeptide (**50**) gave the free side-chain amino group on the Orn residue ready to be derivatised. The conversion of Arg(MC) was attempted by two different approaches. The approach involved a one-step reaction using the known reagent (**53**) to convert the Orn residue directly to Arg(MC). This was unsuccessful, however; 15 eq of the reagent and prolonged reaction time gave poor conversion. The second approach involved a two-step sequence carried out on the Orn residue in (**50**). This involved first guanidination of the side-chain amino function with 1*H*-pyrazole-1-carboxamidine hydrochloride to give the Arg derivative, then acylation with succinimidyl *N*-methylcarbamate in the presence of DBU to give the Arg(MC)-containing intermediate (**51**). The method of introduction of Arg(MC) in this latter step was thus essentially the same as that used in the first reported synthesis. Analysis by HPLC on the crude cyclic peptide (**52**) that was obtained after cleavage from the resin revealed 70% of the mono-acylated compound, plus some doubly acylated material and also unreacted starting material.

To complete the synthesis, the *tert*-butyl ester protection at Asp in (**52**) had to be removed independently of cleavage from the resin in order to avoid aspartimide formation. The treatment of the resin-bound peptide (**51**) with TFA/CH<sub>2</sub>Cl<sub>2</sub> (1:99, v/v) gave the cyclic peptide (**52**) without any side-chain deprotection. The remaining *tert*-butyl ester was then finally cleaved using 1M aq. HCl. One single HPLC purification had to be done in order to isolate the desired fully deprotected argifin (**16**), in 18% overall yield, over a 17-step sequence.<sup>146</sup>

These results demonstrated that the choice of the resin and also the combination of the protecting groups during the linear peptide assembly was effective to eliminate the issue of aspartimide formation but introduced a potential problem of double acylation in the Arg to Arg(MC) conversion (see (**46**) in Scheme 4).



Aspartimide formation is a well-known problem in the synthesis of Asp-containing peptides and it may occur under acidic or basic conditions, leading to the formation  $\alpha$  or  $\beta$ -peptides,<sup>162</sup> as outlined in Scheme 6.

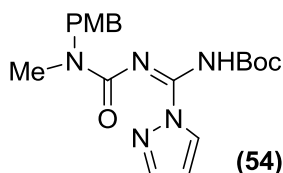


**Scheme 6.** Aspartimide formation from  $\beta$ -Asp peptides.

It appears that argifin (**16**) with its two  $\beta$ -linked Asp residues is particularly sensitive. In this case, the Asp residue followed by D-Ala (“Asp-D-Ala” unit) is probably at most risk of aspartimide formation.<sup>162</sup>

In a subsequent synthesis of (**16**) by solid phase methods, Sunazuka *et al.*<sup>147</sup> developed a specific guanidylating agent (**54**), shown in Figure 26, that can be used to convert an Orn residue directly to Arg(MC) on resin, using a similar protection strategy to that used in Dixon’s synthesis.<sup>146</sup> One minor difference was that in the coupling to the hindered MePhe residue, HATU was used, which proved to be more efficient than PyBOP or PyBrOP. After Fmoc and allyl ester deprotections at the N and C-termini of the linear resin-bound peptide, the cyclisation was carried out in  $\text{CH}_2\text{Cl}_2$  using HATU in the presence of base. Dde was then removed with 2% hydrazine/DMF, followed by the conversion of Orn to Arg(MC) in a one-step reaction. This crucial transformation in the synthesis using (**54**) to modify the Orn side chain gave only a monoacylated Arg intermediate. To complete the synthesis, the protected cyclic peptide was removed from the resin by treatment with 90% TFA in  $\text{CH}_2\text{Cl}_2$  to give (**16**).

This synthetic was achieved in 15-steps from the loading of the first amino acid to resin and gave (**16**) in 13% overall yield.<sup>147</sup>



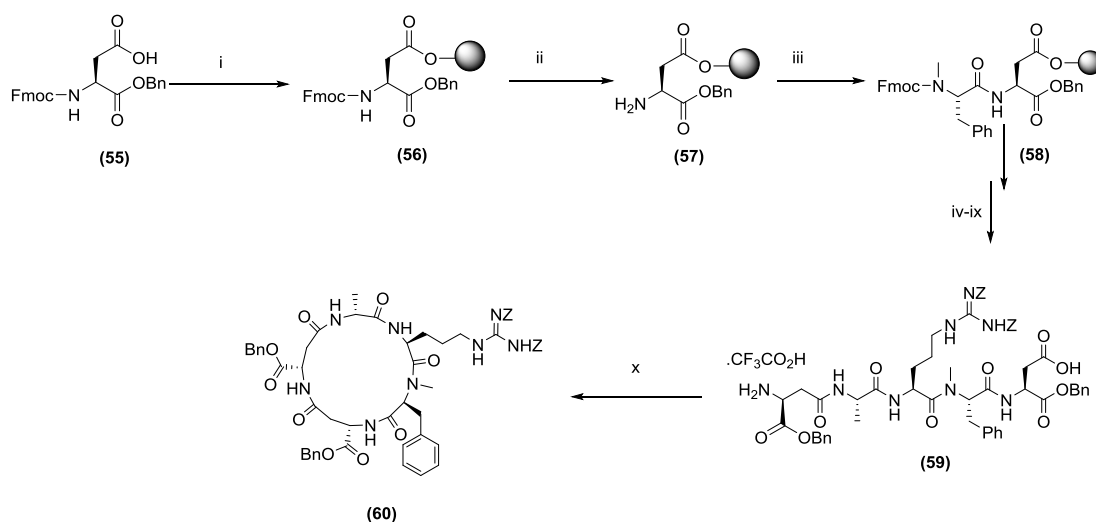
**Figure 26.** Sunazuka's guanidination reagent.<sup>147</sup>

As with the previous reported on-resin synthesis,<sup>143</sup> one single HPLC purification was used. However strategies involving on-resin cyclisation are inherently limited in the scale of peptide that may be synthesised, plus it has been observed that exposure of the final peptide to acidic conditions leads to some risk of aspartimide formation.<sup>162</sup>

The aim was therefore to establish a new approach to (**16**) that is suitable for producing larger quantities of the cyclic peptide, as would be required for *in vivo* studies. To achieve this, the aim was to synthesise a suitable linear peptide by SPPS with cyclisation done by solution chemistry, as in the first synthesis of (**16**).<sup>161</sup> To improve the efficiency of the synthesis, two different approaches were also attempted to introduce the Arg(MC) side-chain, *via* a combination of side-chain protecting groups that should allow avoidance of side reactions seen in previous syntheses.

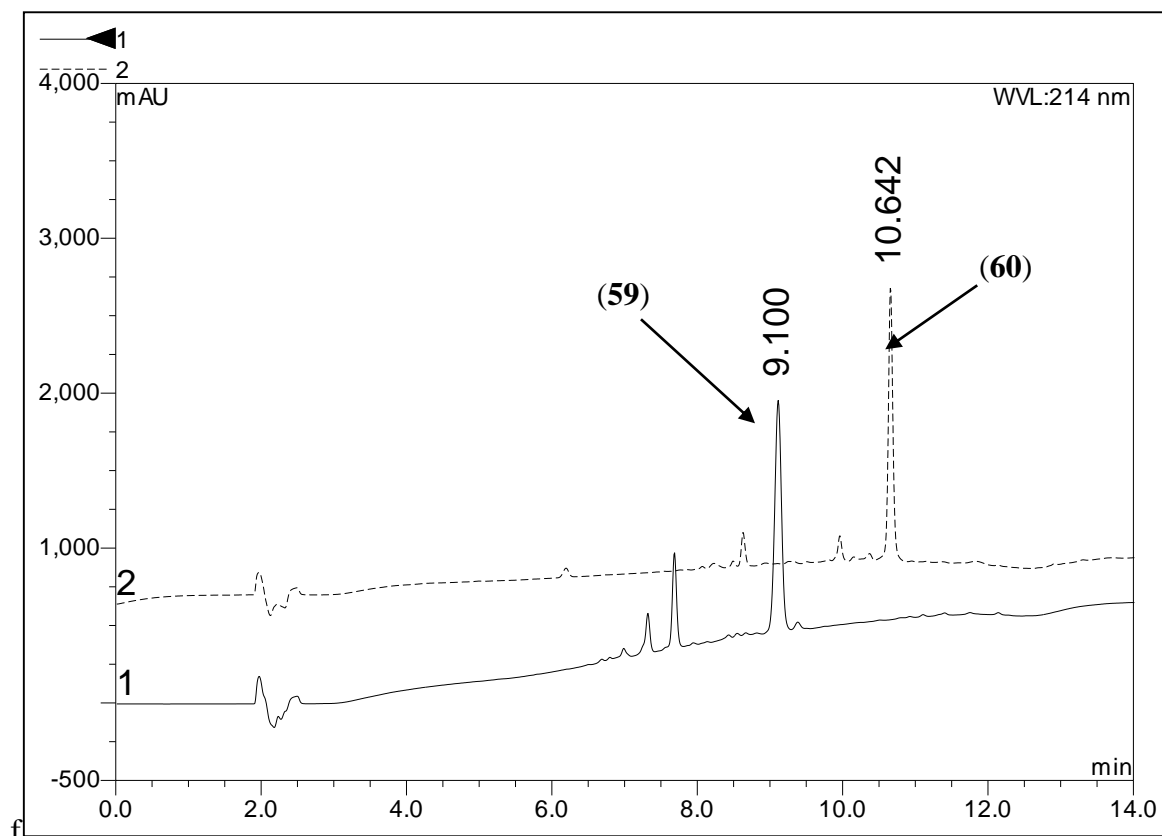
In order to overcome aspartimide formation,<sup>162</sup> benzyl ester and benzyl carbamate (Z) protecting groups were envisaged for the Asp and Arg residues, respectively, which are removable under neutral conditions by hydrogenolysis. As outlined in Scheme 7, the synthesis of the linear precursor peptide (**59**), does not involve the risk of aspartimide formation as the first Asp residue (Asp-OBn) incorporated is directly attached to the solid support (see intermediate (**56**)). Boc-Asp-OBn was chosen to incorporate the second Asp residue at the *N*-terminus for similar reasons. In this way, the *N*-terminal protecting group is removed upon cleavage from the solid support with TFA. This avoids the risk of aspartimide formation which would occur if Fmoc-Asp-OBn was used, where exposure to basic conditions would be required at the final *N*-terminal deprotection step.

For the synthetic approach in Scheme 7, the fully protected linear pentapeptide (**59**) was assembled on Wang resin using 2 eq of Fmoc-protected amino acids and PyBOP (1.9 eq) activation and DIPEA as base, except for the coupling to the hindered MePhe residue where HATU (2 eq) was used. This proved to be more efficient than PyBrOP.<sup>158</sup> The cleavage of the linear peptide from the resin and removal of the Boc protecting group from the N-terminus was achieved with TFA/H<sub>2</sub>O/CH<sub>2</sub>Cl<sub>2</sub> (95:2.5:2.5, v/v/v) to give (**59**) in quantitative yield, based on the calculated resin loading. The cyclisation of (**59**) was then performed in dilute CH<sub>2</sub>Cl<sub>2</sub> solution using HATU activation and DIPEA as base and gave the cyclic pentapeptide (**60**) in 55% yield. The cyclisation proceeded efficiently to give exclusively (**60**), without the need for further purification, as judged by HPLC, shown in Figure 27.



*Reagents and conditions:* (i) Wang Resin DIC, DMAP, CH<sub>2</sub>Cl<sub>2</sub>/DMF (1:1, v/v), 90 min; (ii) piperidine/DMF (1:4, v/v), 4 x 3 min; (iii) Fmoc-MePhe-OH, PyBOP, DIPEA, DMF, 90 min; (iv) Fmoc-Arg(Z)<sub>2</sub>-OH, HATU, DIPEA, DMF, 90 min; (v) piperidine/DMF (1:4, v/v), 4 x 3 min; (vi) Fmoc-D-Ala-OH, PyBOP, DIPEA, DMF, 90 min; (vii) piperidine/DMF (1:4, v/v), 4 x 3 min; (viii) Boc-Asp-OBn-OH; (ix) TFA/H<sub>2</sub>O/CH<sub>2</sub>Cl<sub>2</sub> (95:2.5:2.5, v/v/v), 1 h; (x) HATU, DIPEA, DMF, 48 h.

**Scheme 7.** Improved synthesis of argifin – synthesis of protected cyclic peptide (**60**).

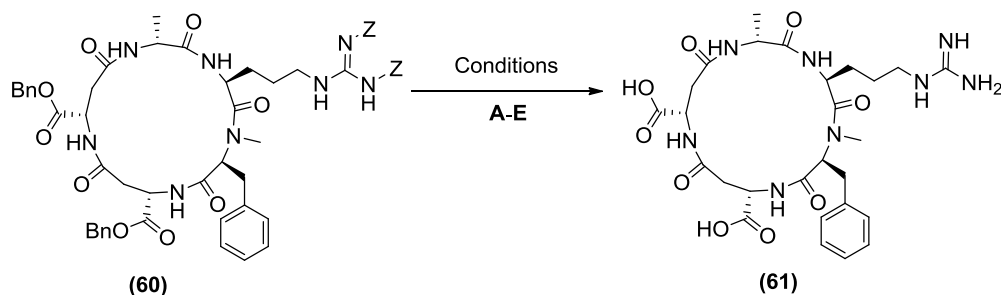


**Figure 27.** HPLC trace of isolated cyclic pentapeptide (**60**) and the linear peptide (**59**).

In summary, the careful choice of alternative protecting groups for the Asp and Arg residues in this synthesis allowed us to obtain the linear peptide (**59**) effectively, which could be cyclised directly with no further HPLC purification required (Figure 27).

A further positive feature of our modified approach is the replacement of the moisture-sensitive 2-chlorotrityl resin, with the cheaper, more robust Wang resin for the linear SPPS.

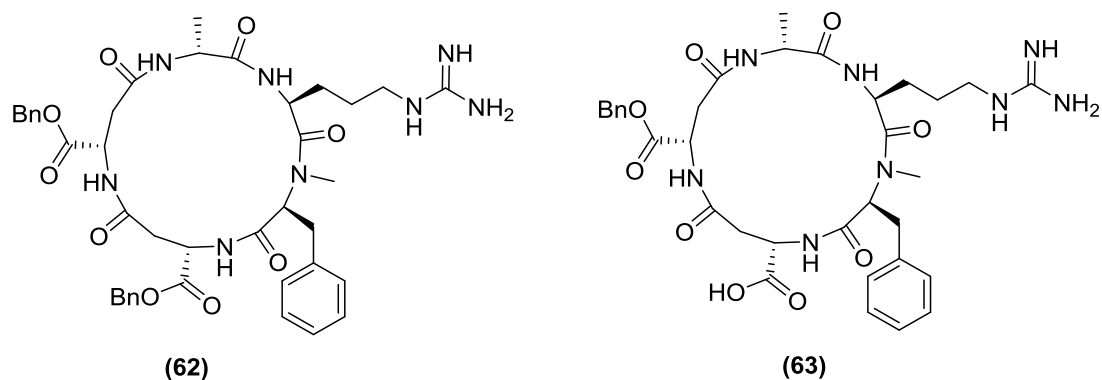
With the fully protected cyclic peptide (**60**) in hand, the removal of all the hydrogenolysable protecting groups of the Asp and Arg residues was examined in detail, as shown in Scheme 8.



*Reagents and conditions:* **A:** H<sub>2</sub>, Pd(OH)<sub>2</sub>/C, AcOH/*i*PrOH, r.t., 1 h; **B:** H<sub>2</sub>, Pd black, AcOH/*i*PrOH, r.t., 18 h; **C:** H<sub>2</sub>, Pd(OH)<sub>2</sub>/C, AcOH/*i*PrOH, r.t., 16 h.; **D:** H<sub>2</sub>, Pd(OH)<sub>2</sub>/C, AcOH/*i*PrOH, r.t., 18 h; **E:** H<sub>2</sub> (4 bar, ), Pd(OH)<sub>2</sub>/C, AcOH, r.t., 72 h.

**Scheme 8.** Hydrogenolysis of (60).

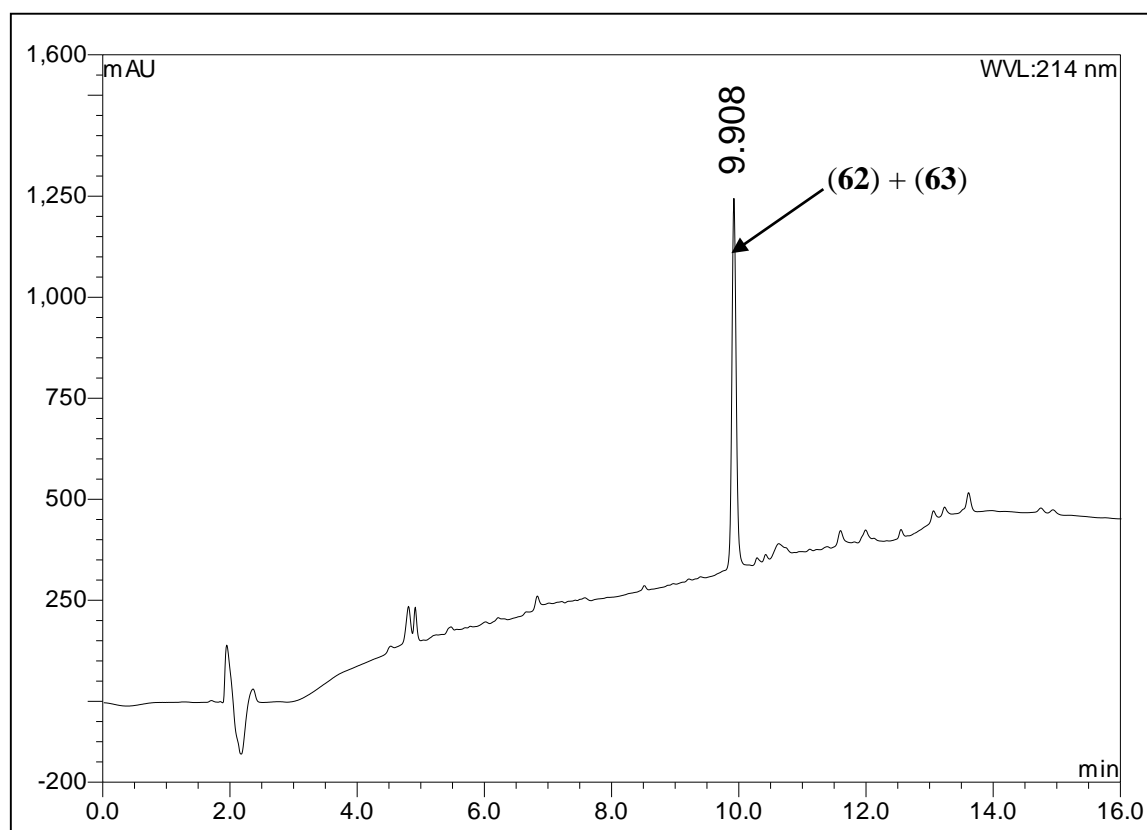
First, the fully cyclic protected peptide (60) was submitted to hydrogenolysis, with Pd(OH)<sub>2</sub>/C in AcOH/*i*PrOH and 1 atm of hydrogen. The reaction was monitored by TLC and, after disappearance of the starting material, analysis by HPLC showed a single new peak at 9.83 min, but mass spectrometry showed the presence of two different compounds (62) and (63), shown in Figure 28. These corresponded to the full deprotection of the Arg residue (removal of both Z groups), with either both benzyl ester protecting groups intact as in (62), or one benzyl ester removed as in (63).



**Figure 28.** Compounds resulting from the hydrogenolysis of protected cyclic peptide (60).

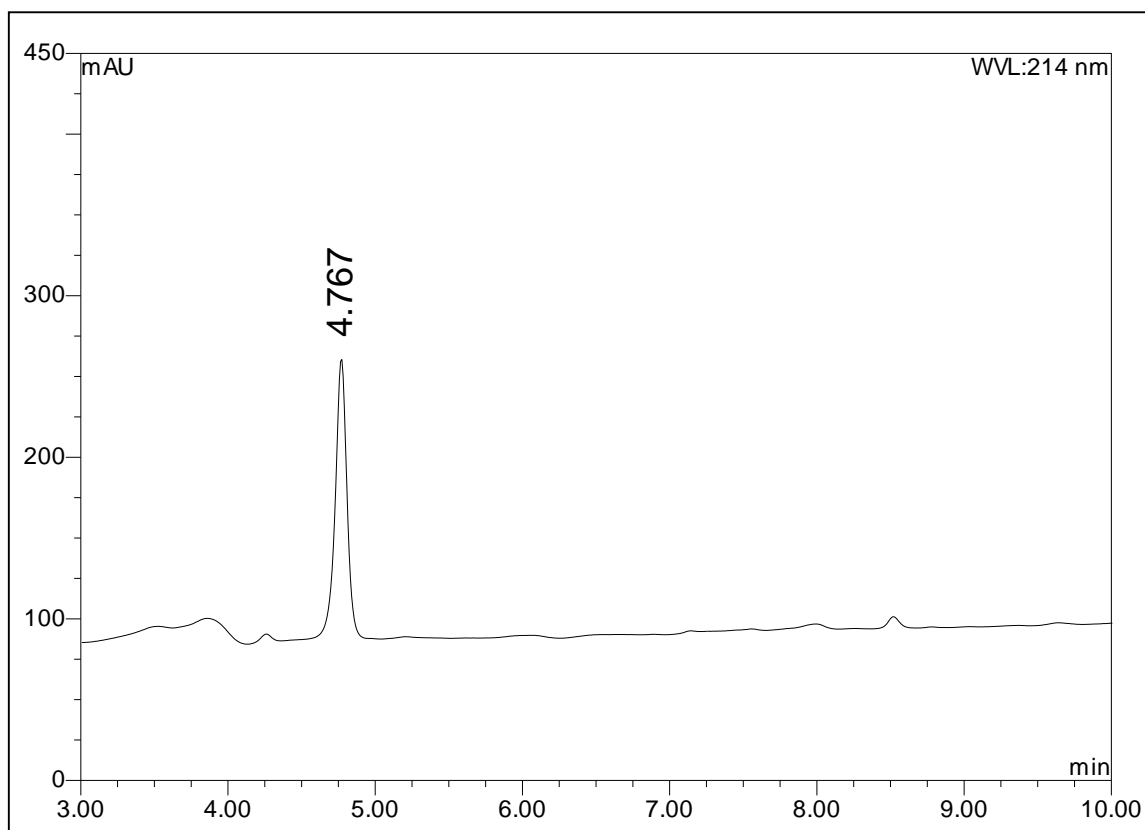
The mixture of the two cyclic peptides (62) and (63) obtained was again submitted to hydrogenolysis, using another equivalent of Pd(OH)<sub>2</sub>/C but no further change was detected by HPLC.

It was speculated that the use of a more reactive catalyst, such as palladium black, should improve the hydrogenolysis; therefore, the same sample was submitted to hydrogenolysis, with this catalyst, under a hydrogen balloon. Once again, no further change was observed. Although the removal of the Z protection was expected to occur more easily than the benzyl esters, the difficulty to achieve full deprotection was unexpected.<sup>163</sup> Repeating the initial deprotection with a fresh sample of the cyclic peptide (**60**) and new catalyst again gave a mixture of (**62**) and (**63**), which were inseparable on HPLC (Figure 29).



**Figure 29.** HPLC trace of the partially deprotected cyclic pentapeptides (**62**) and (**63**).

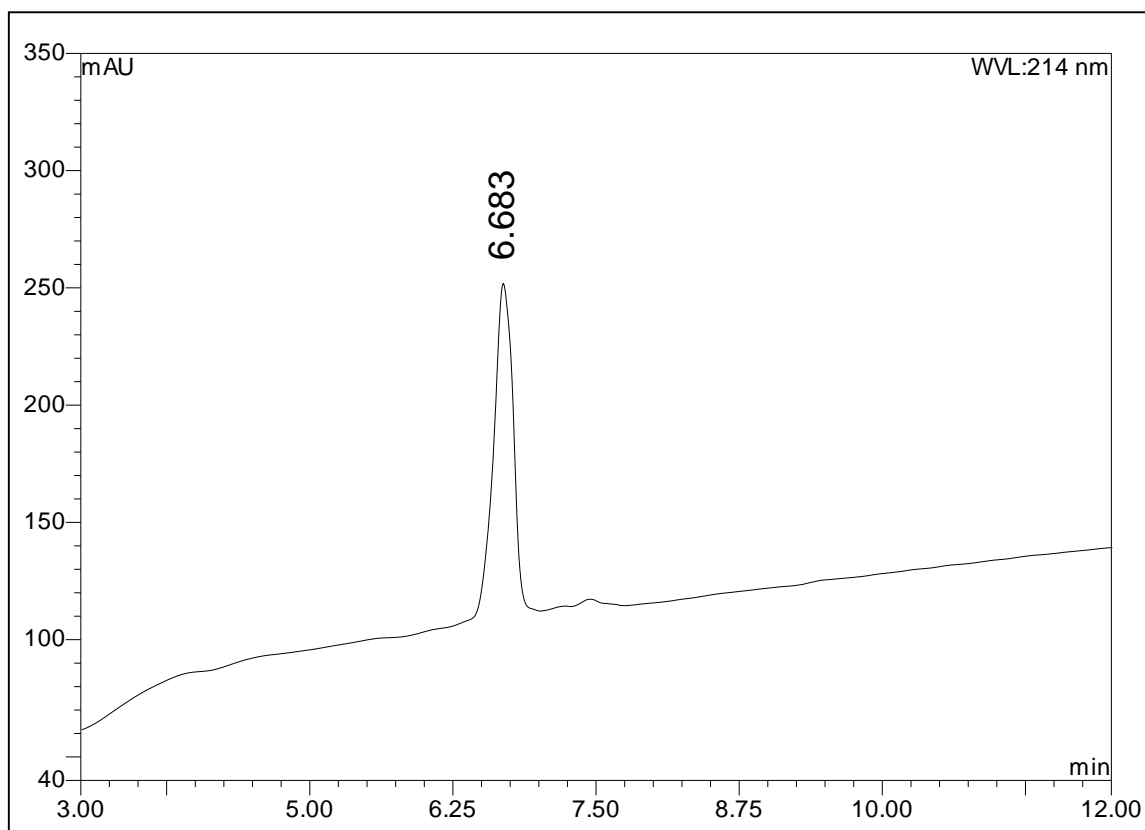
Full deprotection of (**60**) was finally achieved using  $\text{Pd}(\text{OH})_2/\text{C}$ , in AcOH under medium hydrogen pressure (4 bar). HPLC analysis after 72 h now showed a clear conversion to a single new product (**61**) (Figure 30) which was confirmed as the fully deprotected cyclic peptide (**60**) by mass spectrometry.



**Figure 30.** HPLC trace of the fully deprotected cyclic pentapeptide (**61**).

To complete the synthesis, the introduction of the *N*-methylcarbamoyl group onto the *N*<sup>0</sup> position of the Arg side chain was performed according to the method used in Dixon's first synthesis of argifin.<sup>161</sup> The compound (**61**) was treated with 3 eq of succinimidyl *N*-methylcarbamate in DMF at 40 °C in the presence of DBU as base. This gave (**16**) in 19% overall yield from (**61**), after purification by preparative HPLC, as shown in Figure 31.

The final yield of argifin from the fully protected cyclic peptide (**61**) is a slight improvement on Dixon's original approach (19% against 18%). Importantly, only one HPLC purification is required for this isolation of the final product (**16**), by avoiding the use of the acid-labile protection.



**Figure 31.** HPLC trace of argifin (**16**).

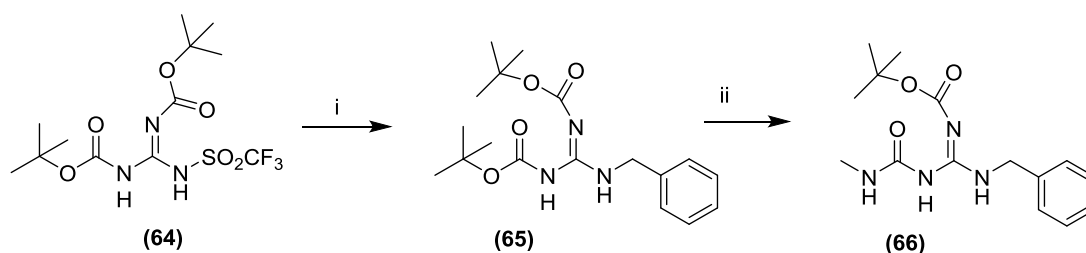
### 5.1.2. Introducing the guanylurea group of argifin; strategy avoiding the double acylation

A critical step in all published syntheses of argifin to date is the introduction of the guanylurea unit of the natural product. When this is effected by treatment of the Arg residue with an activated ester, there is the potential for double acylation to occur.<sup>146</sup> Sunazuka avoided the problem of the double acylation by conversion of an Orn residue to Arg(MC) with a suitable guanidinylation agent. It was decided to explore an alternative one-step approach to the guanylurea moiety of argifin *via* a doubly protected arginine side chain, Arg(Z)<sub>2</sub>. This would avoid again the use of any acid-labile protection.

In previous work in our laboratory, it had been showed that di-Boc-protected guanidines may be converted to the corresponding guanylureas by aminolysis with methylamine.<sup>118</sup> It was envisaged that this type of reaction could be a way of converting an Arg(Z)<sub>2</sub> side chain into the Arg(MC) unit of argifin in one step, so avoiding the problem of double acylation.



As a first step, the Boc-protected compound, *N,N'*-di-Boc-*N'*-benzylguanidine (**65**) was first prepared according to the method of Feichtinger *et al.*<sup>164</sup> by reaction of benzylamine with 1,3-di-Boc-2-(trifluoromethanesulfonyl)guanidine (**64**) in CH<sub>2</sub>Cl<sub>2</sub>, in the presence of triethylamine (5 eq), for 30 min at room temperature (Scheme 5). This gave (**65**) in 72% yield, which was then converted to (**66**) by aminolysis with methylamine (5 eq) in THF at reflux overnight. Analysis of the reaction mixture by TLC showed the presence of the starting material and two other components. The crude product was purified to give unchanged starting material and the desired guanylhurea (**66**) in 55% yield.



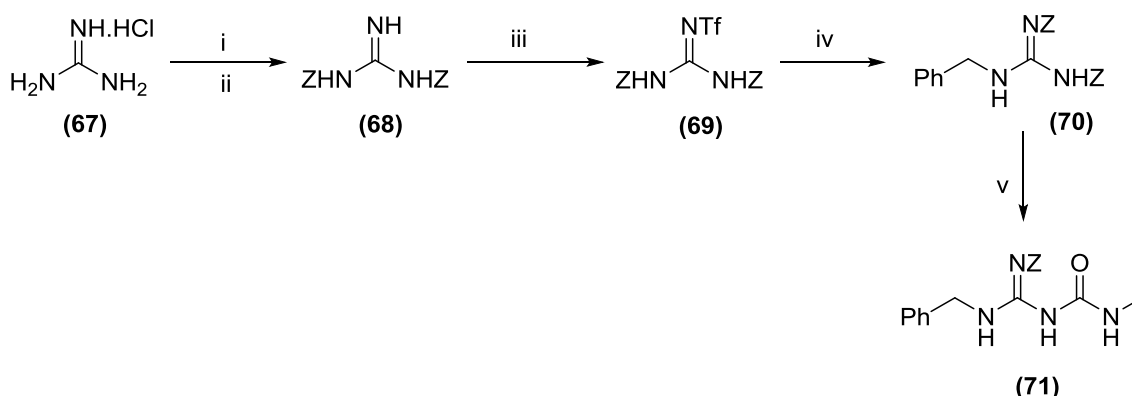
*Reagents and conditions* : (i) Benzylamine (1.1 eq), CH<sub>2</sub>Cl<sub>2</sub>, rt, 30 min, 72%; (ii) MeNH<sub>2</sub> (5 eq), THF, reflux, overnight, 55%.

**Scheme 9.** Aminolysis of the Boc-protected guanidine (**65**) to give guanylhurea (**66**).

In order to investigate the scope of the aminolysis reaction with other carbamate protecting groups, the di-Z guanidine (**68**) was synthesised in an analogous fashion to (**65**). It was first necessary to synthesise the appropriate guanidinyllating reagent (**69**), as described by Feichtinger *et al.*<sup>164</sup> (see Scheme 10). First, guanidine hydrochloride (**67**) was converted to the *N,N'*-di-Z-guanidine derivative (**68**) in 59% yield by treatment with benzyl chloroformate (3 eq) in aqueous sodium hydroxide (5 eq). Next, (**68**) was deprotonated with sodium hydride in anhydrous chlorobenzene at -45 °C, followed by treatment of the resulting anion with triflic anhydride to give (**69**) in 84% yield. The use of chlorobenzene as solvent was essential for the success of this step; the corresponding reaction in THF gave none of the desired product.

With (**69**) in hand, the guanylhurea (**71**) was obtained *via* a two step procedure as before. First, a solution of benzylamine and (**69**) in CH<sub>2</sub>Cl<sub>2</sub> was treated with triethylamine (5 eq) for 1 h at room temperature, to give (**70**) in 81% yield. Compound (**70**) was then treated with methylamine (7.5 eq) in THF at reflux overnight. Analysis of the reaction mixture by TLC

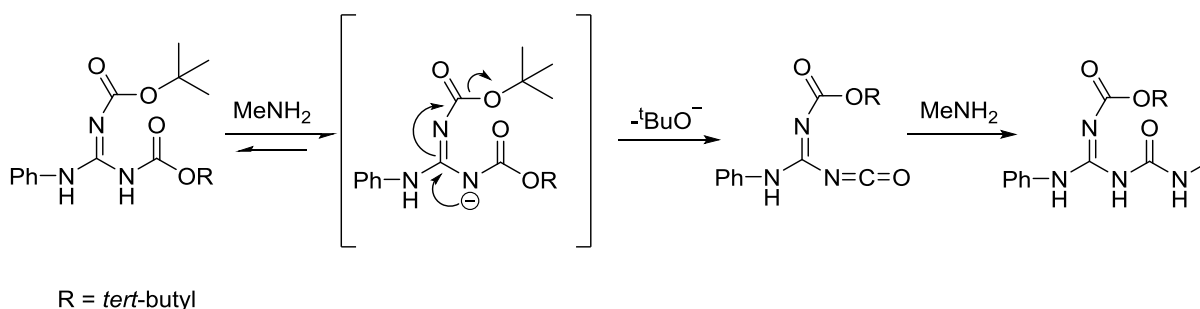
showed the presence of the starting material and two other components. The crude product was purified to give the desired guanylylurea (**71**) in 98% yield.



*Reagents and conditions* : (i) NaOH (5 eq) CH<sub>2</sub>Cl<sub>2</sub>, H<sub>2</sub>O, 0 °C, 1h; (ii) benzyl chloroformate (3 eq), 0 °C -rt, overnight, 59%; (iii) NaH (3.3 eq) chlorobenzene, 0 °C, 1h, triflic anhydride (1 eq), -45 °C -rt, overnight, 84%; (iv) benzylamine (1.1 eq), CH<sub>2</sub>Cl<sub>2</sub>, rt, 1h, 81%; (v) MeNH<sub>2</sub> (7.5 eq), THF, reflux, overnight, 98%.

**Scheme 10.** Aminolysis of the Z-protected guanidine (**70**).

Miel and Rault<sup>165</sup> reported that the conversion of Boc-protected guanidines into ureas with various primary and secondary amines occurs *via* an isocyanate intermediate as shown in Scheme 11. In our case an excess of methylamine was used, which should act both a base to promote the formation of the isocyanate and as a nucleophile, according to Miel and Rault.

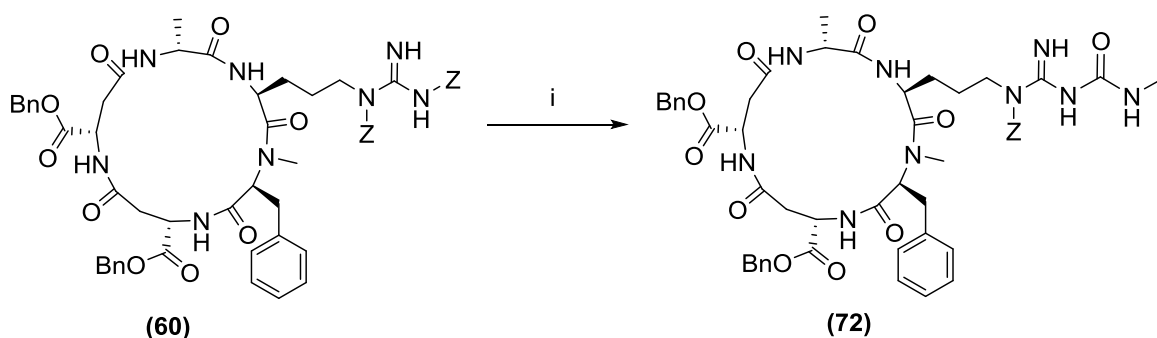


**Scheme 11.** Mechanism of aminolysis *via* an isocyanate proposed by Miel and Rault.<sup>162</sup>

The N-H protons of the di-Boc-protected guanidine are expected to be more acidic than those of the benzylamino unit,<sup>165</sup> due to the possibility of delocalizing negative charge through the guanidine and carbonyl functional groups. The isocyanate intermediate is then generated by the removal of the Boc-protected N-H, followed by the expulsion of a <sup>t</sup>BuO<sup>-</sup> group. If such a

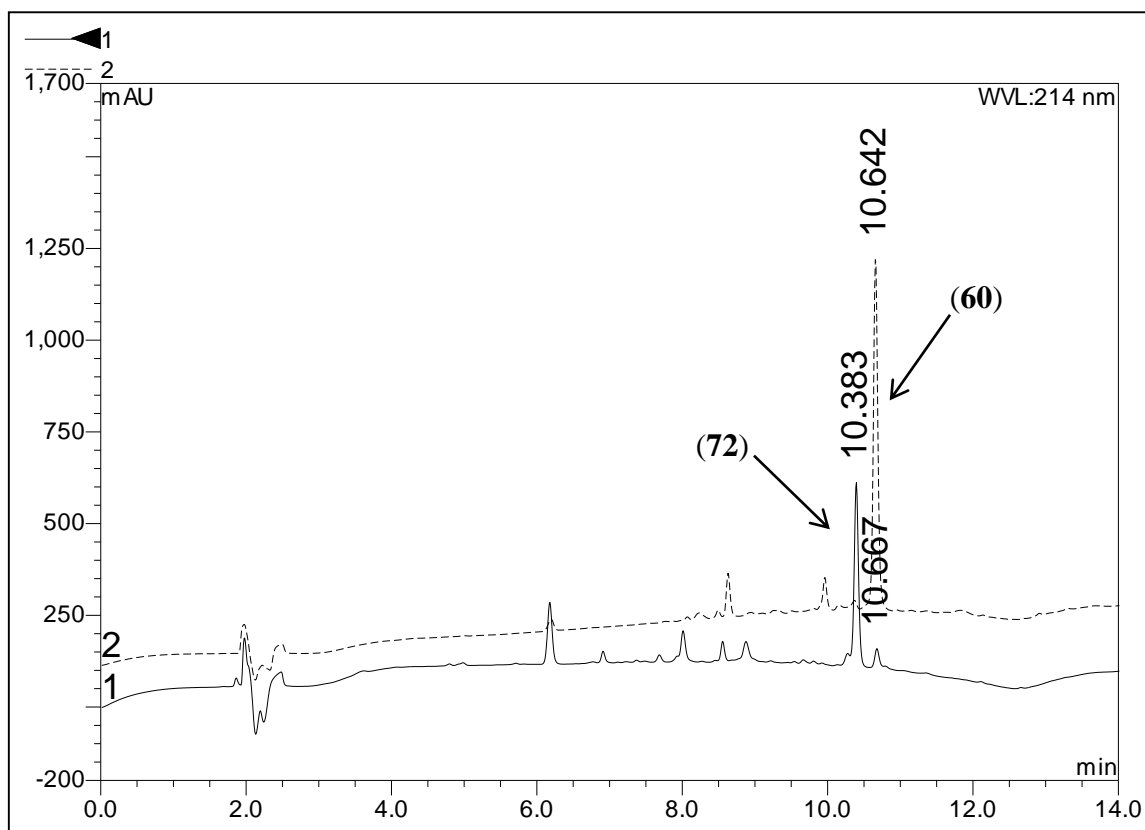
mechanism also operates for *N,N'*-di-*Z*-guanidines such as (**70**), a more efficient conversion (as observed) might be expected, based on the fact that benzyloxy is potentially a better leaving group than *tert*-butoxy, as seen in Scheme 11.

This aminolysis method<sup>165</sup> was then applied to our Arg(*Z*)<sub>2</sub> protected cyclic pentapeptide (**60**) prepared previously. Compound (**60**) was treated with excess methylamine in THF solution and the reaction heated at reflux. After 24 h and 48 h respectively, the reaction was incomplete as shown by HPLC and extra MeNH<sub>2</sub> (5 eq) was added. After 72 h of reaction, a single main product appeared to be formed by HPLC with very little starting material (**60**) remaining (Figure 32). Analysis of the crude reaction mixture by mass spectrometry did not show the formation of the desired product (**72**), but rather a compound *m/z* 933.4 [M+Na<sup>+</sup>] corresponding to the incorporation of two methylamine units with loss of two benzyloxy groups.



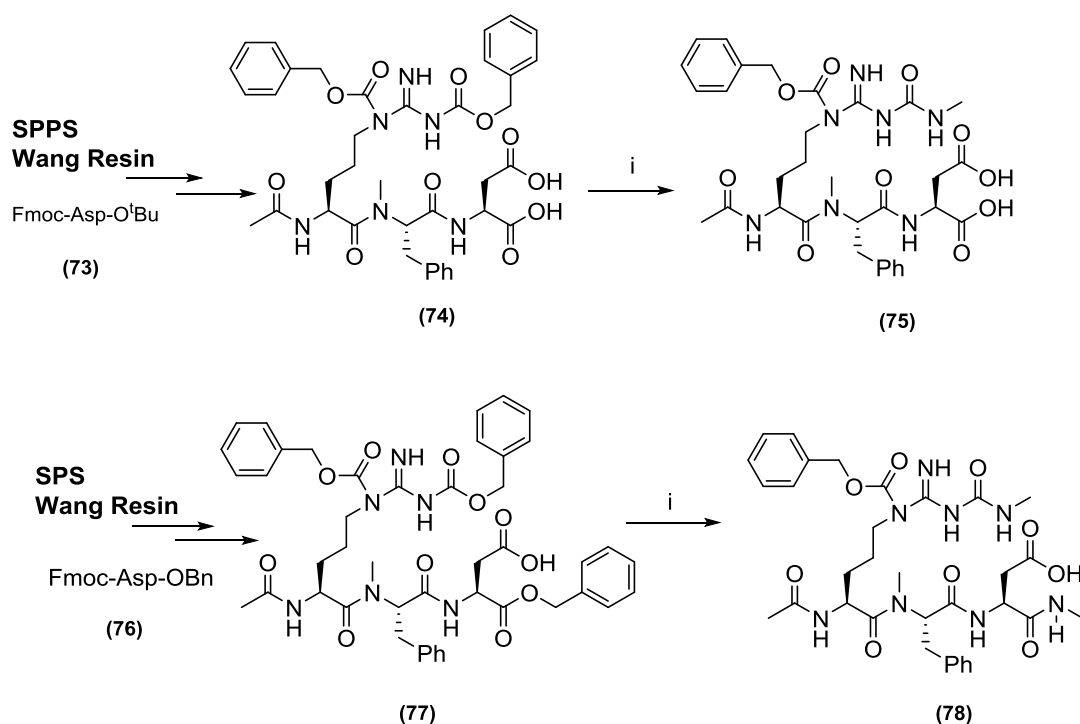
*Reagents and conditions:* (i) MeNH<sub>2</sub>, THF, reflux, 72 h.

**Scheme 12.** Attempted conversion of (**60**) to guanylpentapeptide (**72**), using methylamine.



**Figure 32.** HPLC trace of the aminolysis reaction with **(60)** after 72 h.

In order to investigate whether the aminolysis reaction<sup>165</sup> occurred just at the Arg(Z)<sub>2</sub> side chain or also at aspartic acid, two model tripeptides were prepared. The tripeptide **(74)** may only undergo reaction at Arg, while **(77)** may react at both Arg and Asp (see Scheme 13), to give a product **(78)** with incorporation of two equivalents of methylamine.

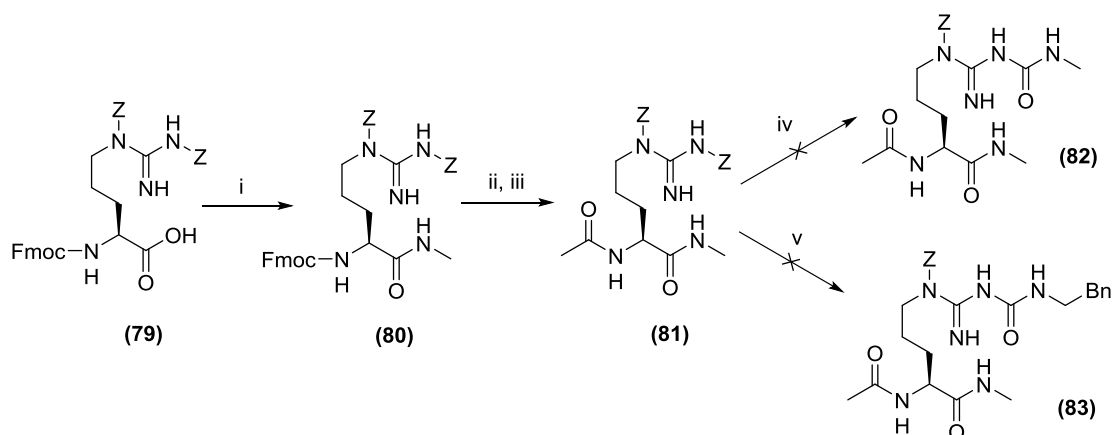


Reagents and conditions: (i) MeNH<sub>2</sub>, THF, 65 °C, 72 h.

**Scheme 13.** Investigation of aminolysis reaction on the tripeptides (74) and (77).

The tripeptides (74) and (77) were synthesised by Fmoc SPPS using Wang resin. These models were then submitted to the aminolysis reaction with 2 eq of MeNH<sub>2</sub> and the reaction was monitored by HPLC. For the reaction of methylamine with (74), HPLC analysis showed no change after 48 h reaction. An excess of 5 eq of methylamine was added to the reaction, but after 72 h the HPLC still indicated that no reaction was occurring. In the case of (77) with MeNH<sub>2</sub> after 48 h reaction, HPLC showed partial consumption of the starting material, therefore another 5 eq of MeNH<sub>2</sub> were added to the reaction mixture but again after 72 h HPLC showed no further change. Although the analysis by HPLC did indicate a new compound, mass spectrometry did not support the formation of the desired compound (78).

Because of the inconclusive results with these models, a simpler argifin-related fragment was chosen for study. It was speculated that the desired aminolysis reaction might be sterically impeded in larger peptide derivatives.



*Reagents and conditions:* (i) EDC.HCl, HOBt, MeNH<sub>2</sub>.HCl, DIPEA, CH<sub>2</sub>Cl<sub>2</sub>, rt, 16 h, 89%; (ii) EtNH<sub>2</sub>, DMF, rt, 30 min; (iii) Ac<sub>2</sub>O, DMAP, CH<sub>2</sub>Cl<sub>2</sub>, 16 h, 78% ; (iv) MeNH<sub>2</sub>, THF, 16 h; (v) BnNH<sub>2</sub>, CHCl<sub>3</sub>, reflux, 16 h.

**Scheme 14.** Synthesis of model compound **(81)** and attempted aminolyses.

The synthesis of this model compound **(81)** is shown in Scheme 14. First, the amide derivative **(80)** was prepared by coupling of **(79)** with methylamine using EDC.HCl and HOBt as activating agents and DIPEA, to give **(80)** in quantitative yield. This was followed by Fmoc deprotection using diethylamine and acetylation with acetic anhydride in the presence of a catalytic amount of DMAP, to give **(81)** in 78% yield. The aminolysis of **(81)** was first attempted in THF with an excess of methylamine but the reaction was unsuccessful and the HPLC indicated the presence of the starting material. The reaction was repeated according to the method of Miel and Rault<sup>165</sup> using CHCl<sub>3</sub> as solvent but this again gave the unchanged starting material. Finally, repeating the reaction with a non-volatile primary amine, benzylamine, also failed to give the desired compound and only the starting material was recovered.

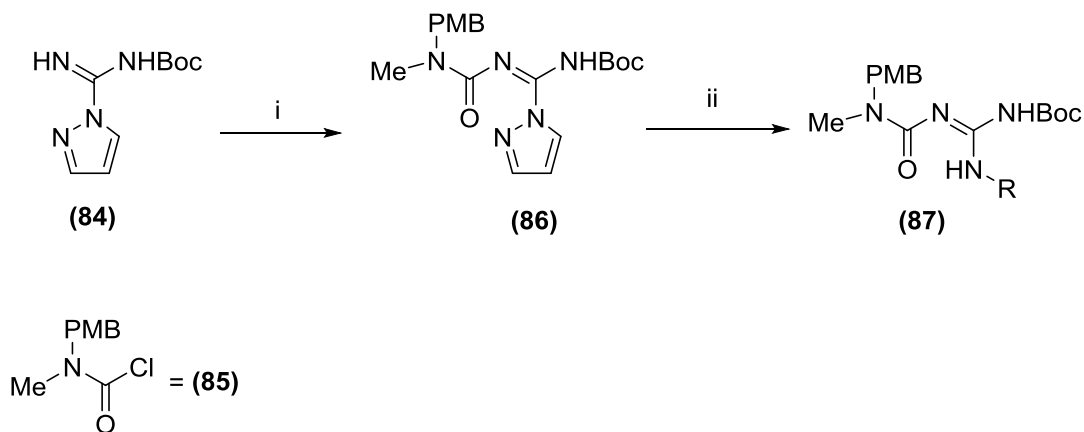
The failure to convert the di-Z-guanidine function of **(81)** into the expected guanylurea with methylamine implies that the reaction may not proceed in the same fashion as for the Boc derivatives. This proposition requires further investigation. In the light of this, it was decided to investigate the preparation of alternative reagents that could be used to effect the conversion Orn→Arg(MC), which would not introduce protecting groups on Arg(MC) that required removal with acid (unlike Sunazuka's original guanidination agent reported<sup>147</sup>).

### 5.1.3. Approaches to alternative reagents for the direct conversion of Orn to Arg(MC)

Guanidine-containing molecules have a wide range of biologically important roles, for example, zanamivir, the influenza inhibitor, possesses a guanidine moiety in its structure.<sup>166</sup>

The most commonly used reagents for the conversion of amines to guanidines include pyrazole-1-carboxamide derivatives,<sup>167</sup> diprotected triflylguanidines and diprotected thioureas,<sup>164</sup> as well as S-methylisothiurea derivatives, which need to be activated by toxic mercury salts<sup>168</sup> or by Mukaiyama's reagent.<sup>169</sup> The choice of guanidinylation reagent usually depends on the synthetic strategy as well as on the reactivity of the starting amine. For reagents such as isothiuronium salts and pyrazoles, it appears that the presence of at least one alkoxycarbonyl group is required on the guanidinylation unit for useful reactivity.

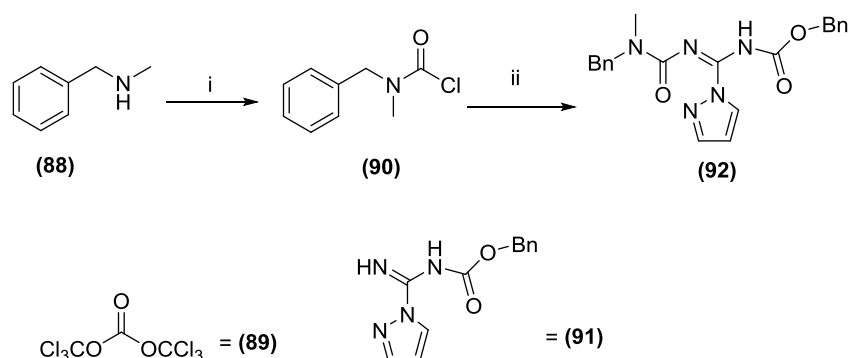
Sunazuka *et al.*<sup>147</sup> reported the synthesis of argifin where the Arg(MC) side chain is introduced on-resin by modifying an Orn side chain. For this they developed a pyrazole reagent (**86**), activated with a Boc group and the acid-labile PMB group, both of which could be removed upon final acidic deprotection of the resin-bound cyclic peptide.



Reagents and conditions: (i) (**85**), NaH, THF, 0-80 °C; (ii) R-NH<sub>2</sub>, Et<sub>3</sub>N, rt.

**Scheme 15.** Pyrazole-based guanidinylation reagent (**87**) developed by Sunazuka.<sup>147</sup>

It was decided to modify this approach, to generate instead a pyrazole reagent (**91**) containing all hydrogenolysable protecting groups. Our attempted preparation of such a reagent is shown in Scheme 16.



*Reagents and conditions:* (i) pyridine, CH<sub>2</sub>Cl<sub>2</sub>, N<sub>2</sub>, -78 °C, 2 h; Method A. (ii) (91), NaH, THF, N<sub>2</sub>, 0 °C to rt; Method B. (ii) as A but used new NaH; Method C (ii) (91), DMAP, DIPEA, CH<sub>2</sub>Cl<sub>2</sub>, rt, 16 h.

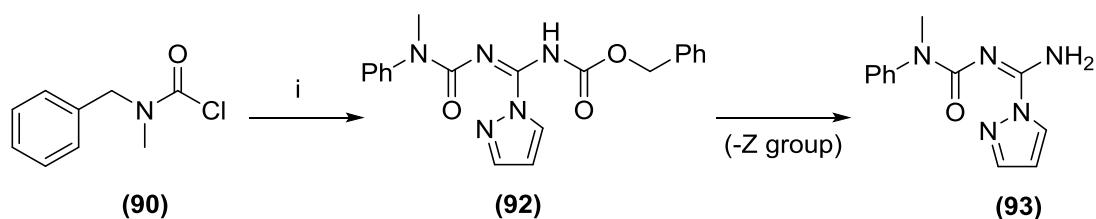
**Scheme 16.** First attempted preparation of pyrazole-based guanidinylation agent (92) with hydrogenolysable protecting groups.

N-Benzyl-N-methylcarbamoyl chloride (90) was first prepared according to the method of Yoakim *et al.*,<sup>170</sup> by the reaction of bis(trichloromethyl)carbonate (89) with benzylmethylamine (88) in the presence of pyridine for 2 h at -78 °C (Scheme 16). This gave (90) in quantitative yield. Conversion to (92) by acylation of the pyrazole derivative (91) was carried out with (90) in the presence of NaH as base in THF. The reaction was carried out at room temperature overnight. Although analysis of the reaction mixture by TLC showed a new component, on purification by analogy with Sunazuka,<sup>147</sup> the two starting materials (84) and (85) were isolated unchanged. Repeating the reaction with a fresh sample of NaH also gave only unchanged starting materials.

An alternative approach was explored in which (90) was further activated with DMAP (0.2 eq) and then reacted with (88) in the presence of base. However this was unsuccessful and analysis by proton NMR was not consistent with the formation of the expected compound.

Since both reactants were recovered unchanged when the reaction with NaH was performed at room temperature, the process was repeated once again at elevated temperature, as shown in Scheme 17.





*Reagents and conditions:* Method **D**: (i) (**91**), NaH, THF, 0 °C to rt, then reflux 16 h.

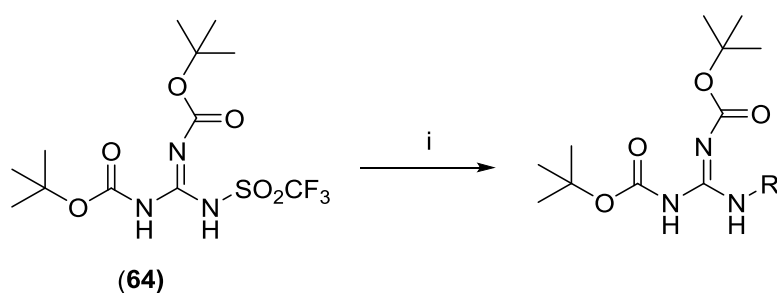
**Scheme 17.** Second attempted preparation of pyrazole-based guanidinylation agent (**93**).

Pyrazole derivative (**91**) was treated with (**90**) and NaH in THF at reflux for 16 h. In this case, analysis of the reaction mixture by TLC showed a new component, which was isolated by column chromatography and shown by  $^1\text{H}$  NMR to be the acylated pyrazole (**93**) lacking the Z protection (Scheme 17).

It seems likely that under these conditions, the expected product is formed but is then decomposed *via* an isocyanate intermediate. Hirose *et al.*<sup>145</sup> have in fact subsequently reported the preparation of (**92**) by these methods but in very low yield (19%).

Due to the difficulty in obtaining the desired pyrazole reagent (**92**), it was decided to explore the preparation of related reagents based on the diprotected triflylguanidine reagents and triurethane guanidines reported by Feichtinger.<sup>164</sup>

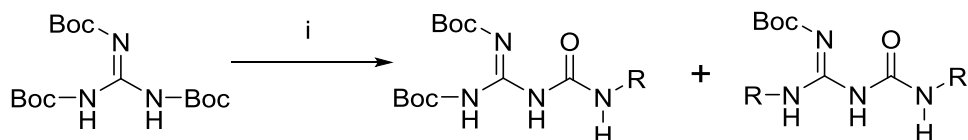
Goodman has shown that reagents such as (**64**) react effectively with a range of amines to give protected guanidines, as shown Scheme 18.<sup>171</sup>



*Reagents and conditions:* (i)  $\text{R-NH}_2$ ,  $\text{CH}_2\text{Cl}_2$ .

**Scheme 18.** Guanidination of amines with di-Boc triflyl guanidine<sup>171</sup>

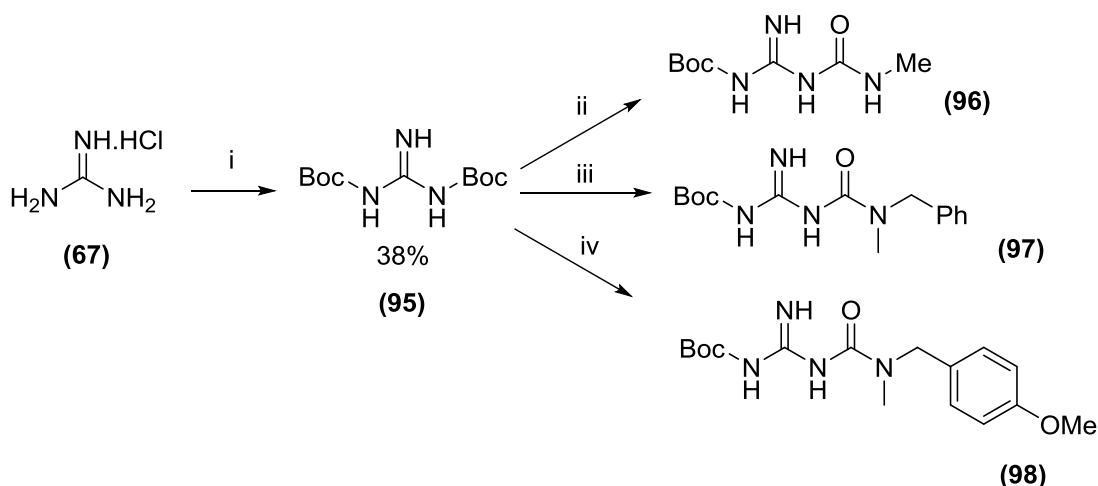
Triprotected urethanes have been less exploited but a report by Prabhakaran and Sanjayan suggests that these compounds may be selectively converted to guanidines or guanylureas, with one or two equivalents of an amine, respectively.<sup>172</sup>



*Reagents and conditions:* (i) R-NH<sub>2</sub>, CH<sub>2</sub>Cl<sub>2</sub>.

**Scheme 19.** Formation of guanidines or guanylureas from tri-Boc guanidine.

Precursors for Goodman-type guanidinylation reagents were prepared as outlined in Scheme 20.<sup>171</sup> These molecules all contain a guanylurea unit, which may then be potentially activated for reaction with various amines.



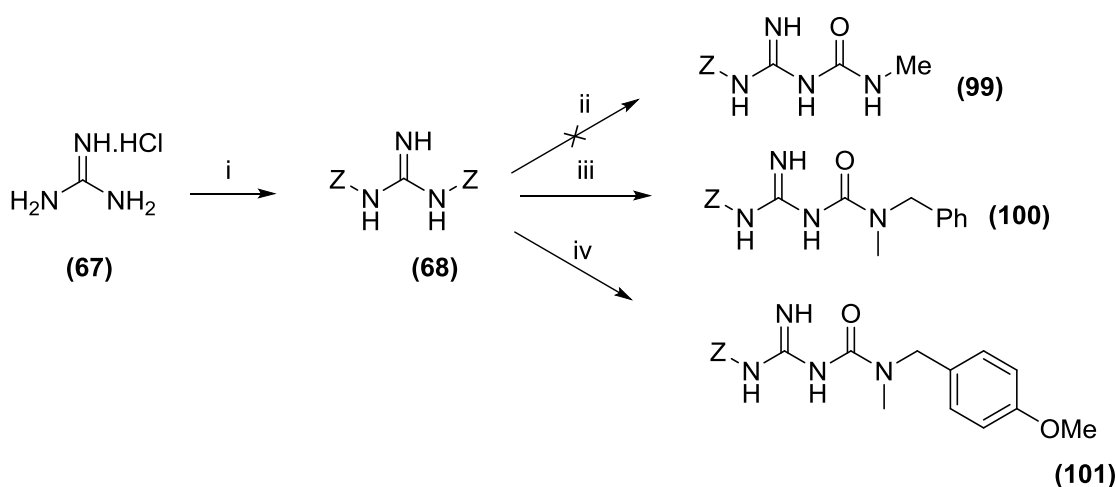
*Reagents and conditions:* (i) Boc<sub>2</sub>O, NaOH, H<sub>2</sub>O, 1,4-dioxane, 0 °C-rt, 16 h, 22%; (ii) MeNH<sub>2</sub>, THF, reflux 16 h, 4%; (iii) (88), THF, reflux, 16 h, 76%; (iv) 4-methoxybenzylmethylamine (94), THF, reflux, 16 h, 86%.

**Scheme 20.** Boc-protected guanidine derivatives.

The di-Boc guanidine (95) was prepared from guanidine hydrochloride and Boc anhydride in a one-pot procedure, to give (95) in 22% yield plus the unchanged starting material. Having already synthesised the di-Boc guanidine, it was hoped that we could then prepare the corresponding guanylureas with a range of methylamine derivatives. For this (95) was first treated with 10 eq of MeNH<sub>2</sub> in THF at reflux for 16 h giving (96) in a low yield of 4%.

Promising results were obtained when **(95)** was treated with only 3 eq of benzylmethylamine **(88)** in THF at reflux for 72 h; this gave the guanylurea **(97)** in 78% yield. The best results were obtained when **(95)** was treated with 3 eq of **(94)** in THF at reflux for 16 h, to give **(98)** in 86% yield, as outlined in Scheme 20.

We also wanted to investigate the same set of reactions with Z-containing compounds, as this could give a guanidinyllating agent that would generate a protected guanylurea that might be deprotected under neutral conditions. This would be particularly useful for the synthesis of argifin.



*Reagents and conditions:* (i) Benzyl chloroformate, NaOH, H<sub>2</sub>O, 0 °C–5 °C, 16 h, 59%; (ii) MeNH<sub>2</sub>, THF, reflux, 18 h 30 min; (iii) **(88)**, THF, reflux, 16 h, 9%; (iv) **(94)**, THF, reflux, 16 h, 13%.

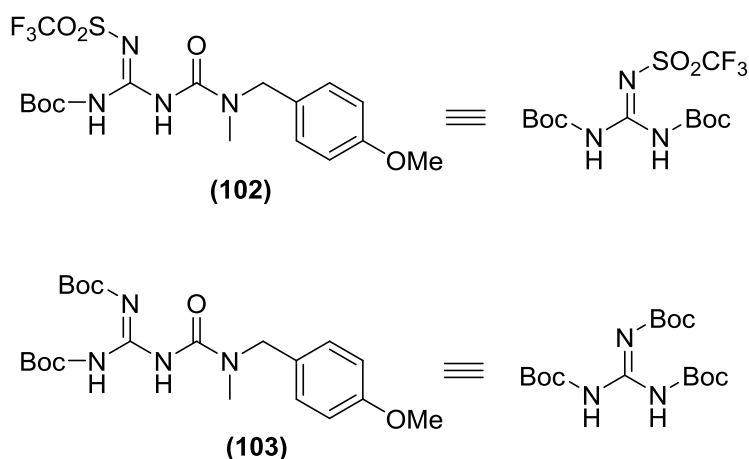
**Scheme 21.** Synthesis of guanylureas **(99)**–**(101)**: precursors for guanidinyllating agents.

The di-Z-guanidine derivative **(68)** was prepared from guanidine hydrochloride and benzyl chloroformate under basic conditions, to give **(68)** in 59% yield. As for the di-Boc-guanidine **(64)**, it was hoped that **(68)** could be converted to the corresponding guanylureas by a reaction with methylamine or a benzylamine derivative. The attempted preparation of **(99)** was first carried out by treating **(68)** with 5 eq of methylamine in THF at reflux for 16 h. After 2 h 30 min reaction, TLC indicated only the starting material, so another 2.5 eq of methylamine were added to the reaction mixture. After 16 h reaction, TLC showed no change and <sup>1</sup>H NMR of the crude product indicated only starting material. It was hypothesized that the volatile amine methylamine was not efficiently delivered in order for the reaction to take place but on repeating the process with 10 eq of methylamine in THF at reflux for 16 h, this again was not successful and <sup>1</sup>H NMR indicated the starting material once more. In contrast, when **(68)** was treated with

10 eq of (**88**) in THF at reflux for 16 h, the reaction did proceed and gave the guanylurea (**100**), although in very poor yield (9%), plus the unchanged starting material in 72% yield. Finally when (**68**) was treated with only 3 eq of (**94**) in THF at reflux for 16 h, this gave (**101**) in 13 % yield plus the unchanged starting material (**68**) (66%) as outlined in Scheme 21.

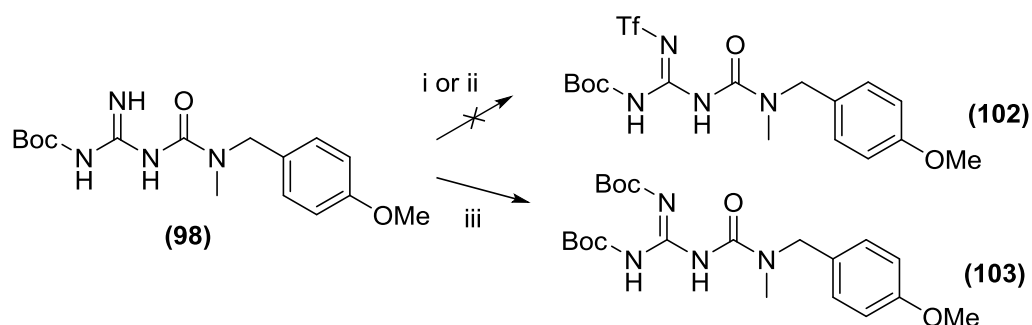
In summary, reactions of the di-Boc-guanidine (**95**) with a methylamine equivalent were more effective than for the corresponding di-Z-guanidine (**68**) with yields ranging from 4% to 86% yield. In the case of using an amine with an electron-rich, alkyl substituent (methoxybenzyl), the highest yield was obtained (86%).

We subsequently investigated the conversion of (**98**) into the corresponding di-Boc (**103**), or 1-Boc-3-triflate (**102**) derivatives (Scheme 22), which would be analogues of the guanidinyllating agents described by Goodman and Prabhakaran.<sup>171, 172</sup>



**Figure 33** (**102**) and (**103**); potential reagents for the direct conversion of amines to protected methylguanylureas.

For the attempted preparation of (**102**), (**98**) was treated with NaH in chlorobenzene at 0 °C and then triflic anhydride was added at -45 °C to the reaction mixture, which was allowed to warm up to room temperature for 16 h. Although the TLC showed the consumption of the starting material, analysis by <sup>1</sup>H NMR was not consistent with the formation of the desired compound, as shown in Scheme 22.



*Reagents and conditions:* (i) NaH, chlorobenzene, 0 °C, 1 h, then -45 °C Tf<sub>2</sub>O, 16 h ; or (ii) DIPEA, CH<sub>2</sub>Cl<sub>2</sub>, then Tf<sub>2</sub>O, -45 °C to rt, 16 h; (iii) Boc<sub>2</sub>O, DIPEA, CH<sub>2</sub>Cl<sub>2</sub>, 0 °C to rt, 14%, 16 h.

**Scheme 22** Attempted preparation of potential guanidinylation agents **(102)** and **(103)**.

It was suspected that the desired product might be unstable under strongly basic conditions and so we substituted DIPEA as base. **(98)** was treated with DIPEA in CH<sub>2</sub>Cl<sub>2</sub> at 0 °C and then triflic anhydride was added at -45 °C to the reaction mixture which was again allowed to warm up to room temperature for 16 h. The <sup>1</sup>H NMR spectrum of the crude product was again not consistent with the expected product.

For the preparation of **(103)**, **(98)** was treated with DIPEA in CH<sub>2</sub>Cl<sub>2</sub> and then Boc anhydride was added at 0 °C and the reaction mixture was allowed to warm up to room temperature and stirred for 16 h. This gave the desired compound **(103)** in 14% yield, plus unchanged starting material (66%), as shown in Scheme 22.

#### 5.1.4. Conclusion and future work:

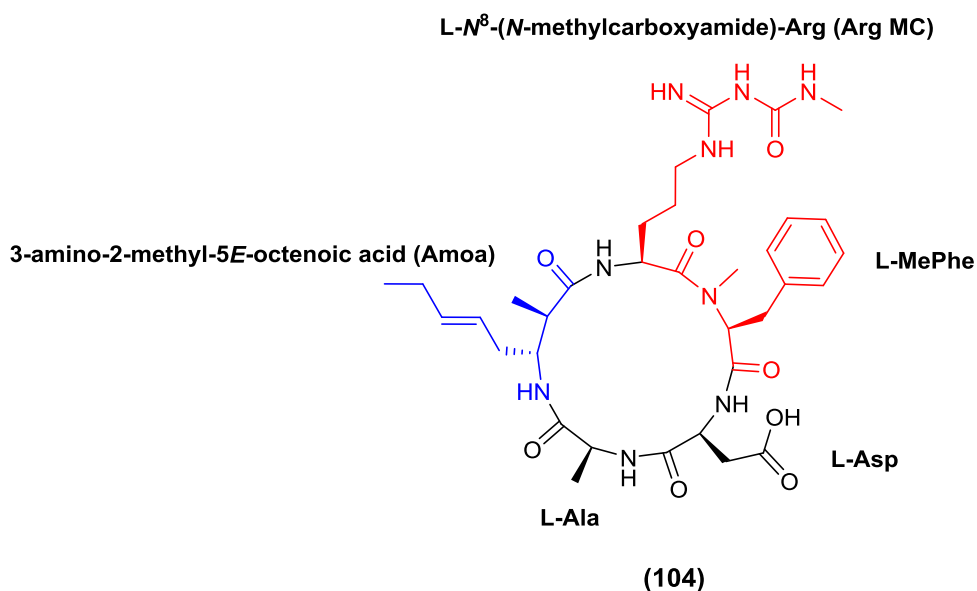
An improved synthesis of argifin has been investigated using a combination of solid phase and solution methods. The use of hydrogenolysable protecting groups avoids the risk of aspartimide formation during final deprotection which is a potential problem in other argifin syntheses. The overall yield by the approach developed here is slightly improved compared to the original synthesis of Dixon *et al.*<sup>161</sup> with only one single HPLC purification being required to isolate the natural product. This method should now be applicable for the large-scale synthesis of argifin for future biological studies.

The preparations of several reagents for the direct conversion of an amine into a guanylurea derivative have been investigated. An analogue (**103**) of the diurethane reagents described by Goodman has been successfully prepared for this purpose. Future work should focus on studying the reaction of various amines with (**103**) to test its usefulness as an agent to introduce the guanylurea unit related to argifin.

## 5.2. Approaches to Amoa: key component of banyasin A

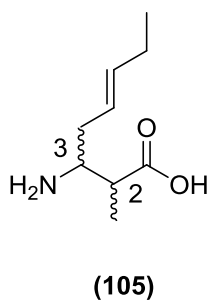
### 5.2.1. Introduction to banyasin A

Banyasin A (**104**) (Figure 34), isolated from the water bloom of the cyanobacterium *Nostoc* sp.,<sup>148</sup> is a cyclic pentapeptide consisting of L-Ala, L-Asp, L-MePhe, L-Arg(MC) and a rare  $\beta$ -amino acid, 3-amino-2-methyl-5*E*-octenoic acid (Amoa). As (**104**) contains the essential Arg(MC)-MePhe dipeptide motif as argifin<sup>142</sup> it would be of considerable interest to synthesise and evaluate banyasin A as a potential Family 18 chitinase inhibitor and to investigate the mode of binding of this molecule to Family 18 chitinases.



**Figure 34.** Structure of banyasin A (**104**).

The stereochemistries configuration of Amoa at C-2 and C-3 in the structure reported by Pluotno and Carmeli are currently unresolved, as outlined in Figure 35.<sup>148</sup>

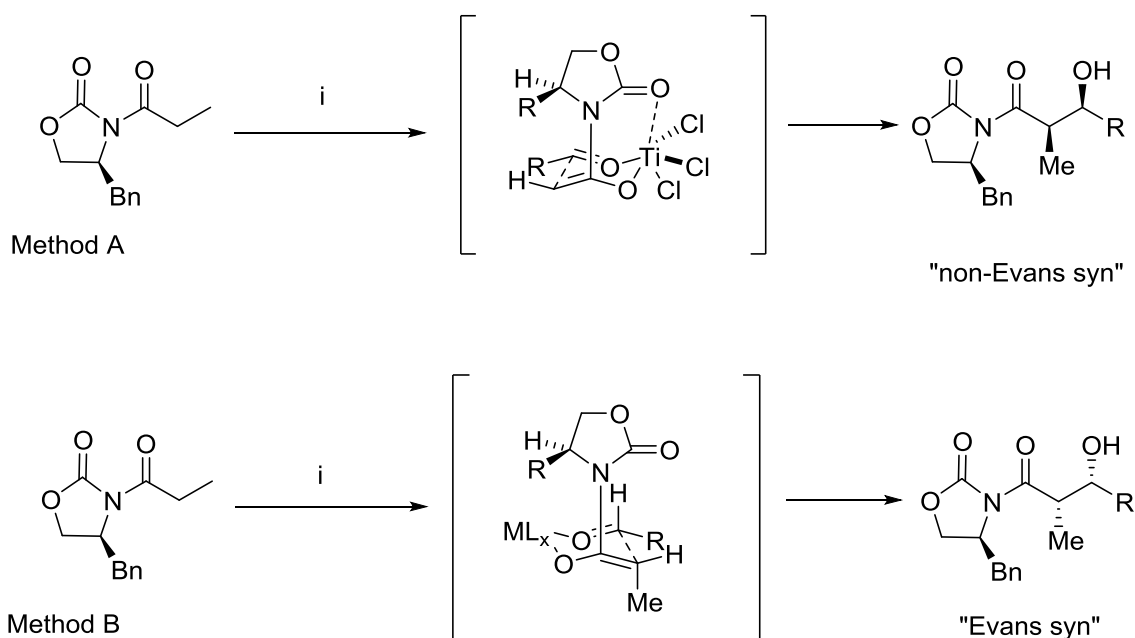


**Figure 35.** 3-Amino-2-methyl-5*E*-octenoic acid (Amoa) (**105**) – the  $\beta$ -amino acid component of banyasin A.

$\beta$ -Methylamino acid derivatives such as Amoa (**105**) occur quite widely in a number of peptide natural products and the syntheses of several examples have been achieved using diastereoselective aldol chemistry.<sup>173</sup>

### 5.2.2. Diastereoselective aldol reactions

The asymmetric aldol addition mediated by chiral auxiliaries is one the most important and general methods for asymmetric carbon-carbon bond formation. Dibutylboron enolates of *N*-acyl oxazolidinones, pioneered by Evans, are the most commonly utilized enolates and are highly effective for the preparation of “Evans syn” products in asymmetric aldol additions.<sup>174</sup>



*Reagents and conditions:* Method A: (i) TiCl<sub>4</sub>, 1 eq amine, RCHO; Method B: (i) TiCl<sub>4</sub>, 2 eq TMEDA or (-)-sparteine.

**Scheme 23.** Transition states to access (A) “non-Evans syn” aldol adducts, (B) “Evans syn” aldol adducts via chlorotitanium enolates.

Crimmins has shown that chlorotitanium enolates of oxazolidinones provide a high diastereoselectivity as well the ability to produce either “Evans” or “non-Evans” syn aldol adduct, depending on the stoichiometry of the TiCl<sub>4</sub> or the nature of the base employed as shown in Scheme (23).<sup>175</sup> Formation of the chlorotitanium enolate of an *N*-acyloxazolidinone with 1.1 eq of TiCl<sub>4</sub> and 2.5 eq of (-)-sparteine followed by the addition of 1.1 eq of the aldehyde



at 0 °C gave the Evans-syn adduct as a major diastereoisomer in high yield with excellent diastereocontrol. High selectivity and excellent yields were also observed when *N*-acyloxazolidinone was enolized with 1.05 eq of TiCl<sub>4</sub>, 1.0 eq of (-)-sparteine and 1.0 eq of *N*-methylpyrrolidinone (NMP). The non-Evans syn aldol adduct was obtained when the enolization was carried out with 2 eq of TiCl<sub>4</sub> and 1 eq of DIPEA. Furthermore the use of 2 eq of TiCl<sub>4</sub>, 1 eq of DIPEA, TMEDA or (-)-sparteine, gave the non-Evans syn adduct. Titanium (IV) enolates of *N*-acyl oxazolidinones and oxazolidinethiones and titanium (II) enolates of thiazolidinethiones have all been shown to have highly organized chelated transition state structures which result in predictable diastereocontrol in reactions with various aldehydes.

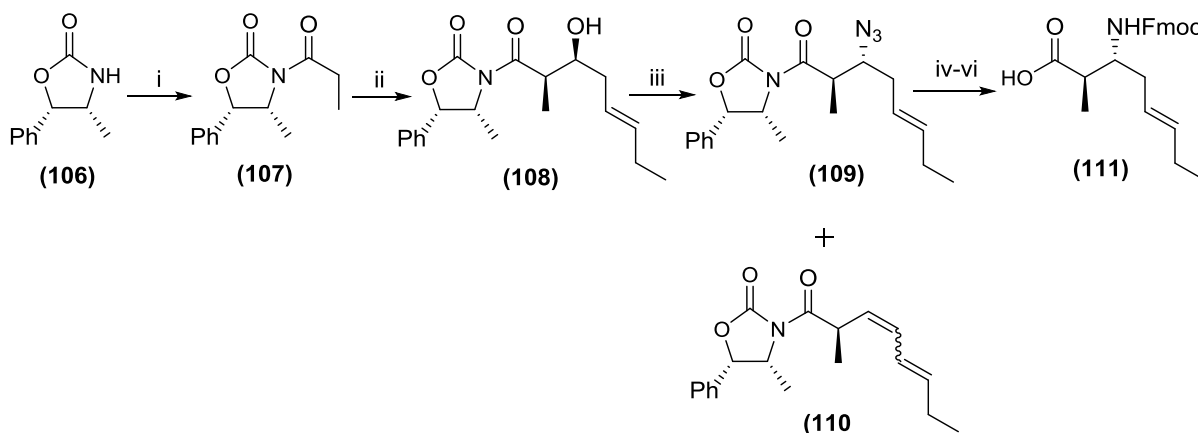
### 5.2.3. Previous approaches to Amoa

In previous work in this laboratory to establish the stereochemistry present in banyasin A (**104**), a strategy for the synthesis of all four stereoisomers of Amoa was devised based on chemistry developed by Crimmins and She.<sup>176</sup> This involves a modified Evans reaction, where the titanium enolate of an optically pure *N*-propanoyl-oxazolidinone was combined with an aldehyde to obtain enantiomerically pure syn aldol products.

Using this approach, one stereoisomer of Amoa (**105**) suitable for application in Fmoc SPPS was obtained as follows. The (4*R*,5*S*)-oxazolidinone derivative (**106**) was acylated with propanoyl chloride, to give *N*-propanoyl derivative (**107**) in 88%. Following Crimmins's procedure,<sup>173</sup> the acylated auxiliary (**107**) was treated with 1.05 eq TiCl<sub>4</sub>, 1.10 eq DIPEA, 1.00 eq NMP and 2.0 eq of hex-3-enal, to give the syn aldol product (**108**) in 72% as a single diastereoisomer. Compound (**108**) was converted into the azide with inverted configuration using a Mitsunobu reaction. The reaction proved to be successful with the use of DPPA as a nucleophilic source of azide, to give the desired azide (**109**) in 34% yield, plus some eliminated material (**110**) in 32% yield. Cleavage of the chiral auxiliary from (**109**) with alkaline hydrogen peroxide, followed by Staudinger aza-Wittig reduction of the azido (**109**) and *N*-protection using Fmoc-OSu, provided the β-amino acid (**111**) in overall 21% over three steps, as outlined in Scheme 24.

It is important to note that the removal of the auxiliary from (**109**), preceded by the reduction of the azido function dramatically increased the yield of the conversion of (**109**) to (**111**).

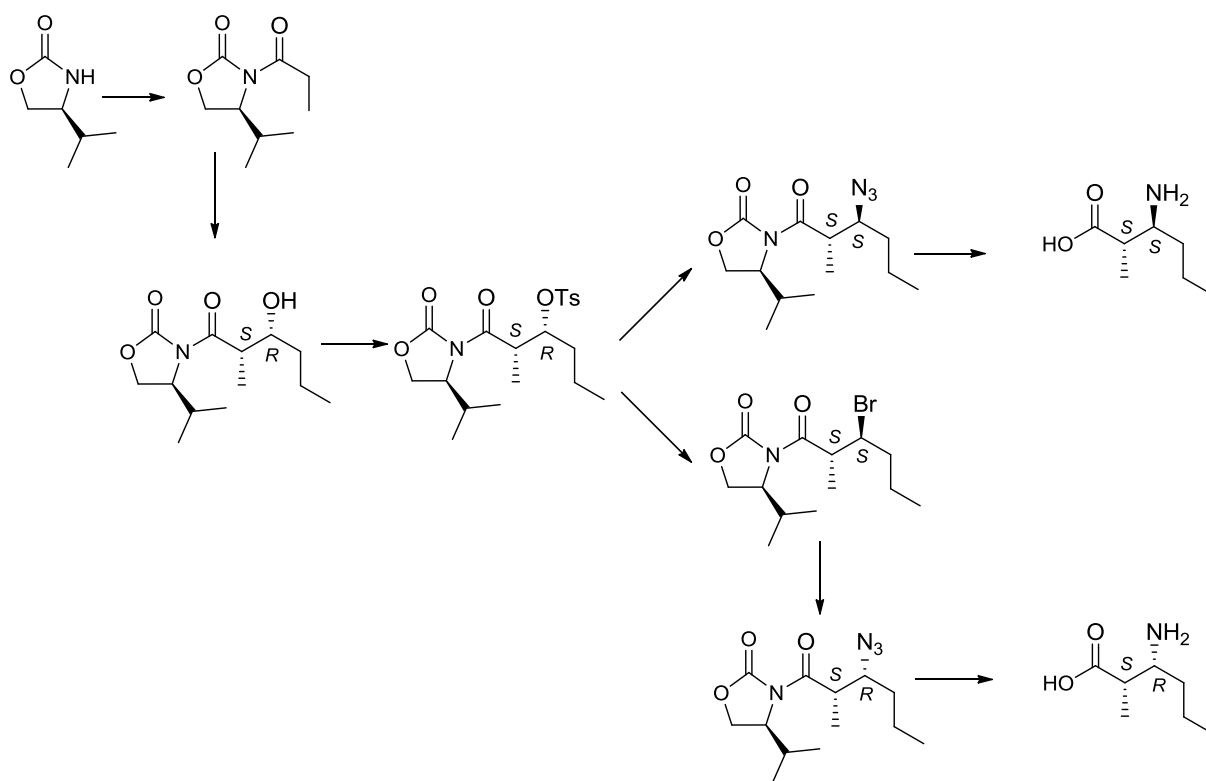
Furthermore using Fmoc-OSu avoided the formation of the mixed anhydride intermediate and ester by-product, which seemed to occur when Fmoc-Cl was used.



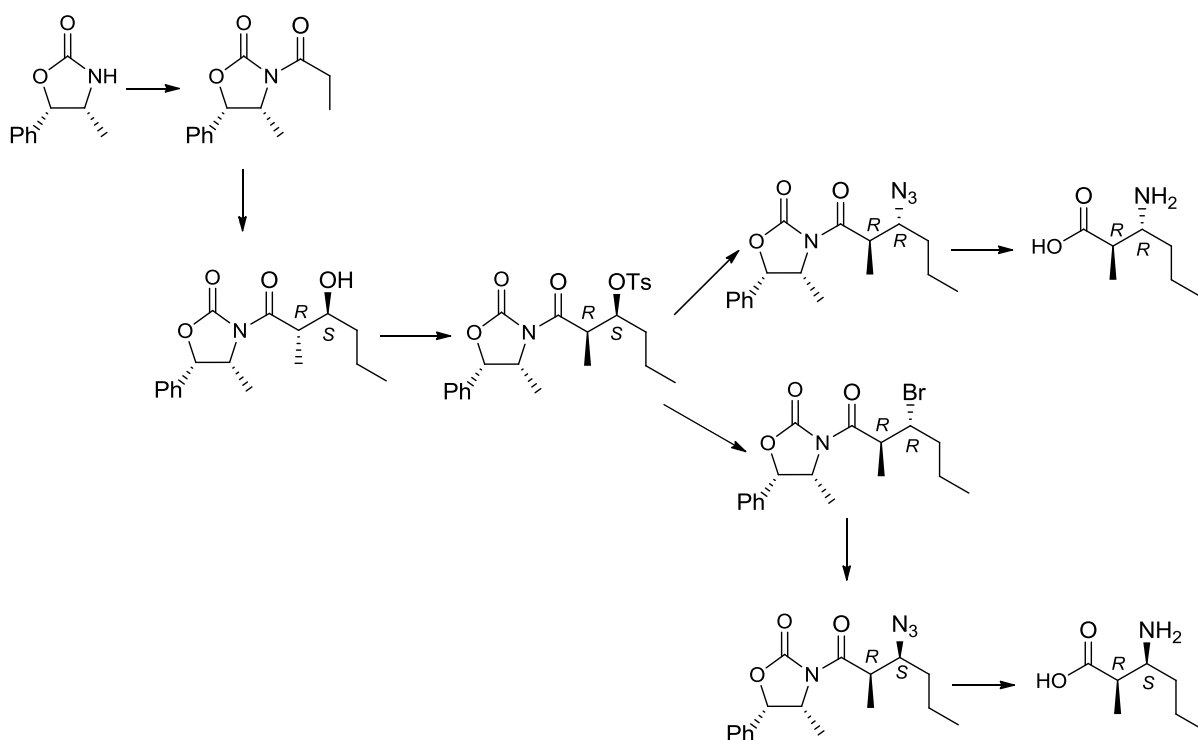
*Reagents and conditions:* (i) *n*-BuLi, propanoyl chloride, N<sub>2</sub>, -78 °C to rt, 16 h, 88%; (ii) TiCl<sub>4</sub>, DIPEA, NMP, hex-3-enal, N<sub>2</sub>, 0 °C, 6 h, 72%; (iii) PPh<sub>3</sub>, DEAD, N<sub>2</sub>, -20 °C for 1 h, DPPA, -20 °C to rt, 16 h, 34%; (iv) LiOH/ H<sub>2</sub>O<sub>2</sub>, 3 h, 0 °C; (v) PPh<sub>3</sub>, N<sub>2</sub>, rt, 30 min then H<sub>2</sub>O, 60 °C, 16 h (vi) Dioxane, 10% Na<sub>2</sub>CO<sub>3</sub>, Fmoc-OSu, 0 °C to rt, 16 h, 21%.

**Scheme 24.** Synthesis of one diastereoisomer of (**105**) based on diastereoselective aldol chemistry.

Following on from Nathubhai's work,<sup>177</sup> we also aimed to prepare all 4 stereoisomers of (**105**) by a strategy used by Kimura in the preparation of kulokekahilide<sup>173</sup> In this work four stereoisomers of 3-amino-2-methylhexanoic acid (**111**) were obtained from two syn aldol products, introducing the N-function with either inversion of configuration or net retention, as shown in Schemes 25 and 26.



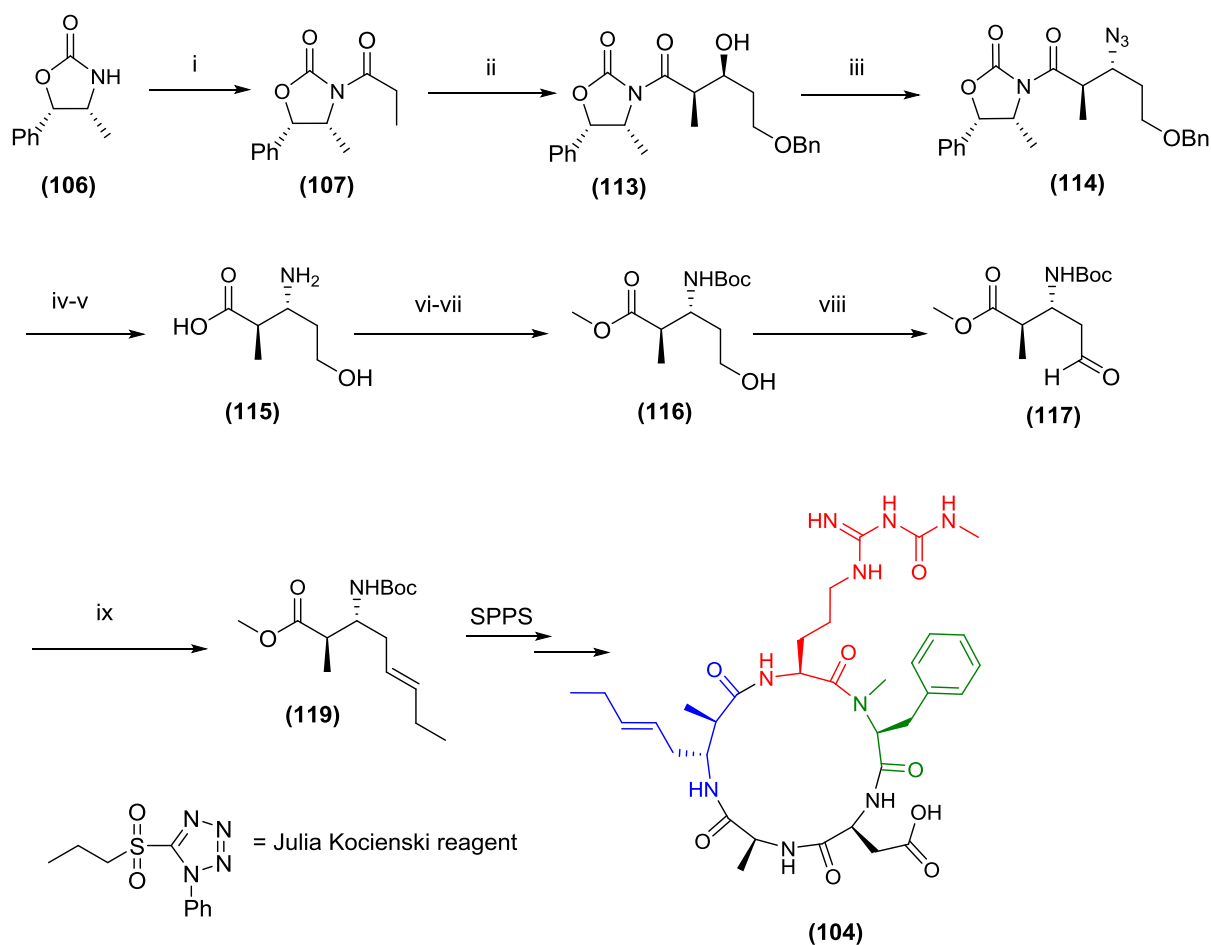
**Scheme 25.** Synthesis of (2*S*, 3*S*) and (2*S*, 3*R*)-3-amino-2-methylhexanoic acids.<sup>173</sup>



**Scheme 26.** Synthesis of (2*R*, 3*R*) and (2*R*, 3*S*)-3-amino-2-methylhexanoic acids.<sup>173</sup>

#### 5.2.4. New approach to Amoa using Evans chemistry

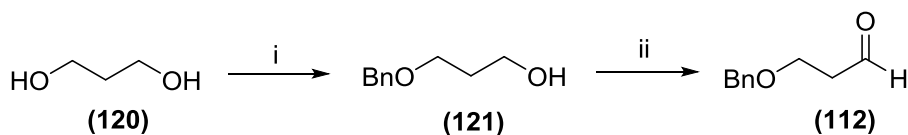
To avoid the problems of elimination observed in Nathubhai's work<sup>177</sup> when an azido function was introduced by Mitsunobu chemistry, we aimed to investigate the introduction of the alkene function of Amoa at the end of the synthesis. The unsaturated chain would be introduced by Julia-Kocienski olefination, after cleavage of the Evans auxiliary as outlined in Scheme 27. Here, (**106**) would be acylated with propanoyl chloride and then the *N*-propanoyl derivative (**107**) would be subjected to a Crimmins aldolization with 3-(benzyloxy)propanal (**112**). The enantiomerically pure (2*R*,3*S*)-aldol product (**113**), would then be converted into the azide (**114**) by inversion of configuration using a Mitsunobu reaction. Cleavage of the chiral auxiliary from (**114**) with alkaline hydrogen peroxide, followed by catalytic reduction of the azido function and debenzylation, would then provide the  $\beta$ -amino acid (**115**) which could be transformed into the Boc-protected derivative (**116**). To complete the synthesis of (**105**), the hydroxyl group would be oxidised to the aldehyde (**117**) by Swern oxidation, followed by the introduction of the alkene function carried out via *E*-selective Julia-Kocienski olefination to give (**119**). Ester hydrolysis and protection of the amino function with the Fmoc group would then give one stereoisomer of Amoa ready to be introduced into standard solid phase peptide synthesis.



**Reagents and conditions:** (i) propanoyl chloride, n-BuLi, THF, -78 °C; (ii) TiCl<sub>4</sub>, DIPEA, (112), NMP, 0 °C-, 7 h; (iii) DPPA, Ph<sub>3</sub>P, DEAD, THF, -20 °C-rt; (iv) H<sub>2</sub>O/THF, LiOH/H<sub>2</sub>O<sub>2</sub>, 0 °C; (v) H<sub>2</sub>, Pd/C; (vi) MeOH, HCl, (vii) Boc<sub>2</sub>O, NaHCO<sub>3</sub>, H<sub>2</sub>O-dioxane; (viii) (COCl)<sub>2</sub>, DMSO, Et<sub>3</sub>N, CH<sub>2</sub>Cl<sub>2</sub>, (ix) (118), Julia Kocienski reagent, KHMDS (0.5 M in DME), DME.

**Scheme 27.** Proposed synthetic approach for the preparation of one single diastereoisomer of (105) - the Fmoc-protected derivative (119)

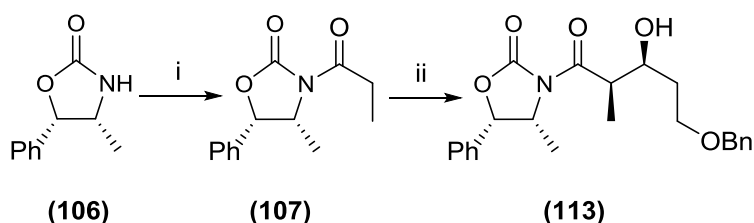
The first stage in our synthetic approach was the preparation of the protected aldehyde (112) from the diol (120), as this aldehyde was not commercially available.



**Reagents and conditions:** (i) Benzyl bromide, TBAI, NaH, THF, 0°C then warming to rt, 16 h, 76%; (ii) (ClCO)<sub>2</sub>, DMSO, 30 min, -78 °C, Et<sub>3</sub>N then warming to rt, 16 h, 24%.

**Scheme 28.** Synthesis of aldehyde (112).

The diol (**120**) was treated with benzyl bromide with TBAI, giving the monoprotected alcohol (**121**) in 76% yield.<sup>178</sup> Compound (**121**) was subjected to Swern oxidation; this was attempted by two different methods. The first attempted involved treating (**121**) with oxalyl chloride and DMSO according to the method of Davis and Qi.<sup>179</sup> This gave the desired aldehyde (**112**) in 24% yield, plus unchanged starting material. Repeating the oxidation according to the method reported by Davis<sup>180</sup> with a more diluted solution of (**120**) in CH<sub>2</sub>Cl<sub>2</sub> gave (**112**) in an improved yield of 46% but still with some unchanged starting material remaining. The aldehyde proved to be unstable at room temperature and decomposed on standing. It was therefore freshly prepared for the aldol chemistry. The aldehyde (**112**) was subjected to the Crimmins aldol reaction<sup>176</sup> with the acylated chiral oxazolidinone (**107**), as shown in Scheme 29.



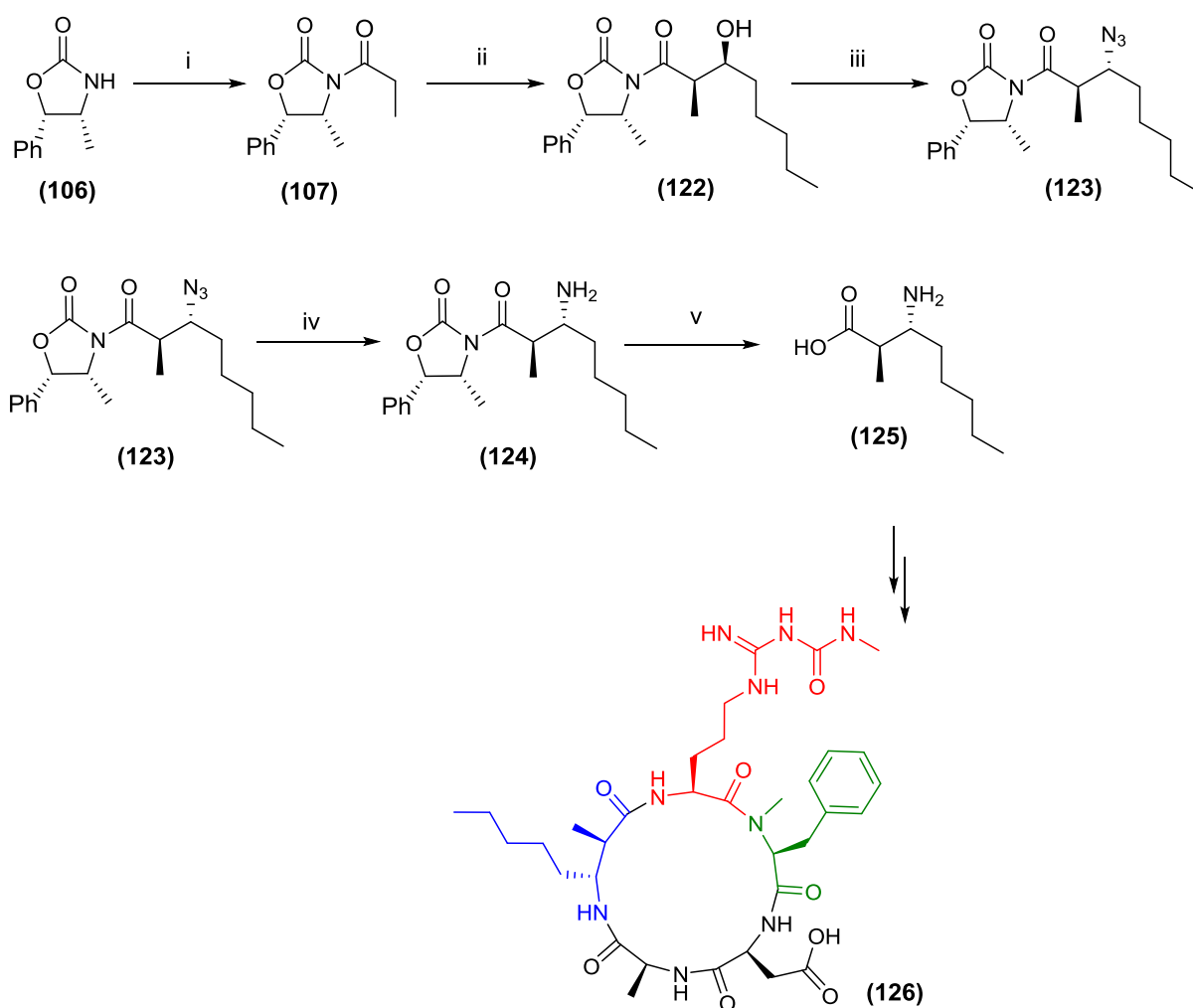
*Reagents and conditions:* (i) propanoyl chloride, n-BuLi, THF, -78 °C to rt, 16 h, 84%; (ii) TiCl<sub>4</sub>, DIPEA, CH<sub>2</sub>Cl<sub>2</sub>, -5 °C, then NMP, (**112**), 4 h, 39%.

**Scheme 29.** Synthesis of the syn aldol product (**113**).

The (4*R*,5*S*)-oxazolidinone derivative (**106**) was acylated with propanoyl chloride, to give *N*-propanoyl derivative (**107**) in 84% yield. The diastereoselective aldol addition reaction between (**107**) and (**112**) was carried out using the optimised Crimmins procedure.<sup>173</sup> The reaction was first effected with 1.05 eq of TiCl<sub>4</sub>, 1.10 eq of DIPEA, 1.00 eq of NMP and 2.00 eq of aldehyde (**112**) at 0 °C, then warming up to room temperature for 16 h. This reaction was unsuccessful and both starting materials were recovered unchanged. Compound (**107**) was again subjected to the Crimmins aldol reaction but this time the titanium enolate was formed at -5 °C and allowed to react with 2 eq of the freshly made aldehyde (**112**) and stirred at -5 °C for 4 h. The aldol reaction proceeded smoothly in this case and gave the (2*R*,3*S*)-aldol product (**113**) in 39% yield, plus both unchanged starting materials. The stereochemical assignment of (**113**) is based on three pieces of evidence. Firstly, the <sup>1</sup>H NMR obtained for (**113**) is identical to that reported by Kigoshi *et al.*,<sup>181</sup> for the same syn aldol product prepared from (4*R*,5*S*)-oxazolidinone (**106**) using Evans boron enolate chemistry. The optical rotation obtained for (**113**) was also consistent with that reported by Kigoshi.<sup>181</sup> Finally, in the <sup>1</sup>H NMR spectrum

obtained for (**113**), the H-2 signal at  $\delta$  3.85 shows the expected dq pattern, with a coupling constant  $J_{H2-H3}$  of 3.8 Hz, which is consistent with the syn aldol configuration. Reports by Mukaiyama and others indicate that in non-polar media,<sup>182, 183</sup> a larger coupling constant should be expected for an anti aldol product (*e.g.* 9 Hz).

Because of its potential biological activity against Family 18 chitinases, we intended to focus our attention on the unsaturated chain of Amoa, in order to determine the importance of this unit within (**105**). Thus in parallel, we planned to investigate the synthesis of some analogues of (**105**), both linear and cyclic, in which the unsaturated side chain would be replaced by a saturated group. This would be achieved using Crimmins aldolization as shown in Scheme 30, since the appropriate required aldehyde hexanal was stable and commercially available. The aldehyde would be subjected to Crimmins procedure to give the enantiomerically pure syn aldol product (**122**), whose hydroxy function could then be converted into an amino group *via*  $S_N2$  chemistry.



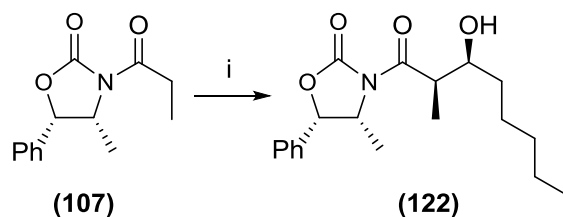
Banyasin with Amoa reduced

*Reagents and conditions:* (i) propanoyl chloride, n-BuLi, THF, -78 °C; (ii) TiCl<sub>4</sub>, DIPEA, NMP, hexanal; (iii) DPPA, Ph<sub>3</sub>P, DEAD, THF, -20 °C-rt; (iv) H<sub>2</sub>, Pd/C (v) H<sub>2</sub>O/THF, LiOH/H<sub>2</sub>O<sub>2</sub>, 0 °C;

**Scheme 30.** Synthetic approach to reduced Amoa analogue (**125**) and banyasin A analogue (**126**).

The diastereoselective aldol addition of the acylated auxiliary (**107**) and hexanal was again carried out under Crimmins optimised conditions.<sup>176</sup> The reaction was effected with 1.05 eq of TiCl<sub>4</sub>, 1.10 eq of DIPEA, 1.00 eq of NMP and 2.00 eq of freshly distilled hexanal, at 0 °C then warming up to room temperature for 7 h. This gave the Evans syn aldol product (**122**) in 74% yield, as shown in Scheme 31.

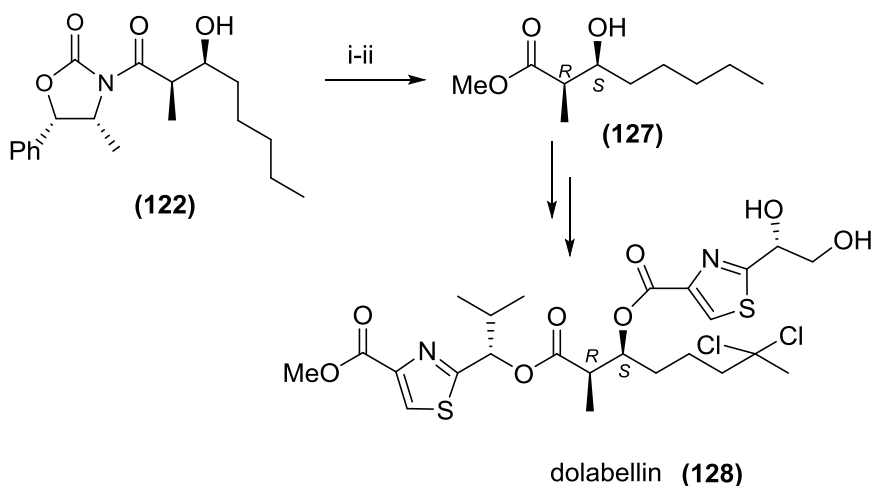




*Reagents and conditions:* (i)  $\text{TiCl}_4$ , DIPEA,  $\text{CH}_2\text{Cl}_2$ ,  $0^\circ\text{C}$ , then NMP, hexanal, 7 h, 74%.

**Scheme 31.** Synthesis of the syn aldol product **(122)**.

The stereochemical assignment of **(122)** could again be confirmed by comparison of the  $^1\text{H}$  NMR and optical rotation of the product obtained with that previously reported by Sone<sup>184</sup> for the same syn aldol that was prepared using Evans boron enolate chemistry. Once again, the  $^1\text{H}$  NMR of **(122)** showed the expected dq at  $\delta$  3.78 for the H-2 signal, with a coupling constant  $J_{\text{H2-H3}}$  of 2.7 Hz, consistent with the syn aldol configuration reported by Sone.<sup>184</sup> In Sone's study,<sup>184</sup> the syn configuration of **(122)** was further supported by conversion to a corresponding  $\beta$ -amino acid whose  $^1\text{H}$  NMR could be compared with a closely related material of known configuration derived from the natural product dolabellin (see Scheme 32).



*Reagents and conditions:* (i)  $\text{LiOH}$ ,  $\text{H}_2\text{O}_2$ , THF,  $\text{H}_2\text{O}$ , rt; (ii)  $\text{CH}_2\text{N}_2$ , ether, rt.

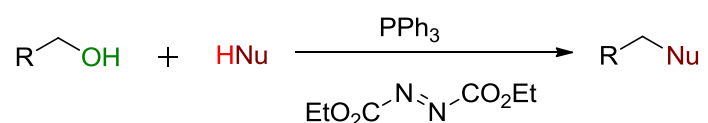
**Scheme 32.** Conversion of **(122)** to dolabellin-derived material.<sup>184</sup>

### 5.2.5. Approaches to Amoa synthesis using displacement reactions for introduction of the amino function

#### *Mitsunobu reactions*

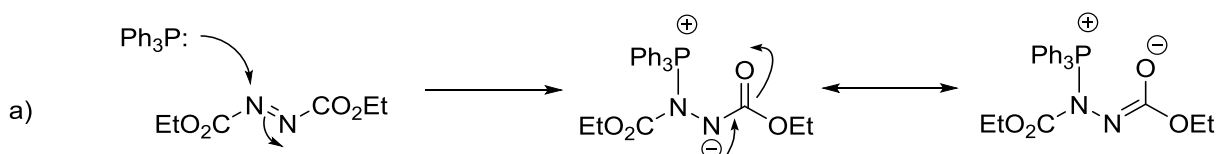
The Mitsunobu reaction<sup>185</sup> allows the conversion of primary and secondary alcohols into esters, phenyl ethers, thioethers and various other compounds. It involves the condensation of an alcohol  $R^1-OH$  and acidic compound  $H-Nu$  to form  $R^1-Nu$ , with the aid of a trialkyl/triarylphosphine reagent and an azodicarboxylate. The nucleophile  $Nu$  used should be acidic, since the azodicarboxylate should be protonated during the course of the reaction, to prevent side reactions. The trialkyl/triarylphosphine combines with an azodicarboxylate (*e.g.* diethyl azodicarboxylate – DEAD) to generate a phosphonium intermediate that deprotonates the hydroxyl function of the alcohol, which is then converted into a good leaving group.

The overall result of a general Mitsunobu reaction is shown in Scheme 33 :

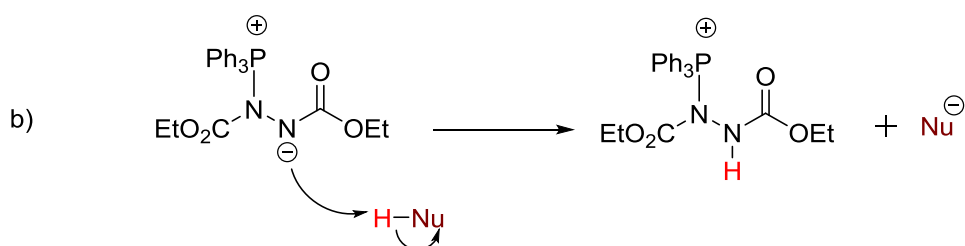


**Scheme 33.** Mitsunobu reaction between an alcohol and nucleophilic species,  $Nu$  ( $Nu = N_3$ ).

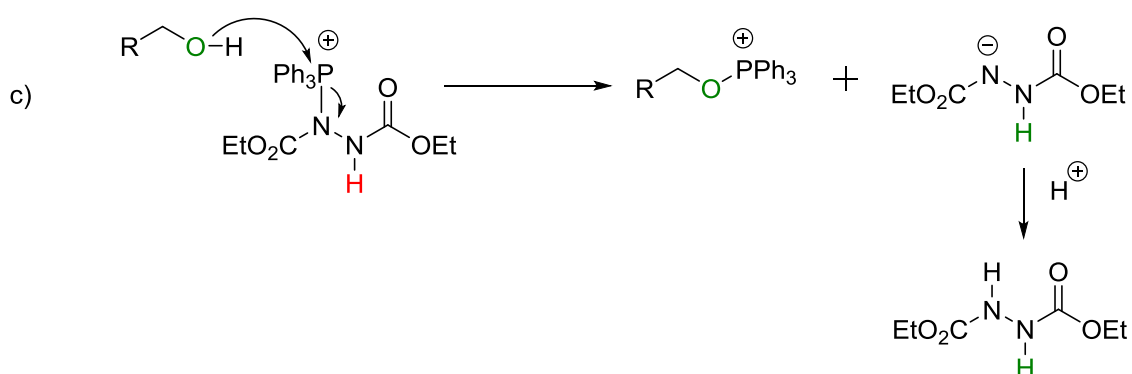
Mechanism of the Mitsunobu reaction:



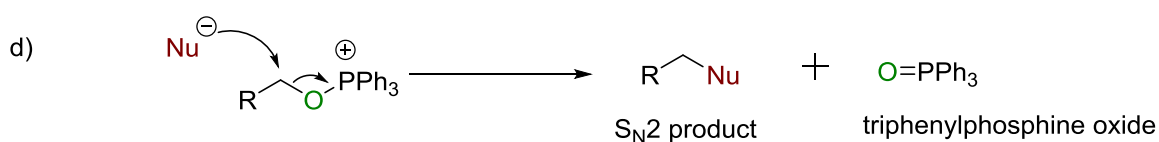
The phosphine reduces the weak  $N=N$  bond of the diethyl azodicarboxylate (DEAD) to give a betaine intermediate. The nitrogen anion produced is stabilized by the delocalization of charge through the ester group, as shown in Scheme 34 step a.



The betaine intermediate removes a proton from the nucleophile, to give an ion pair, as shown Scheme 34 in step b.



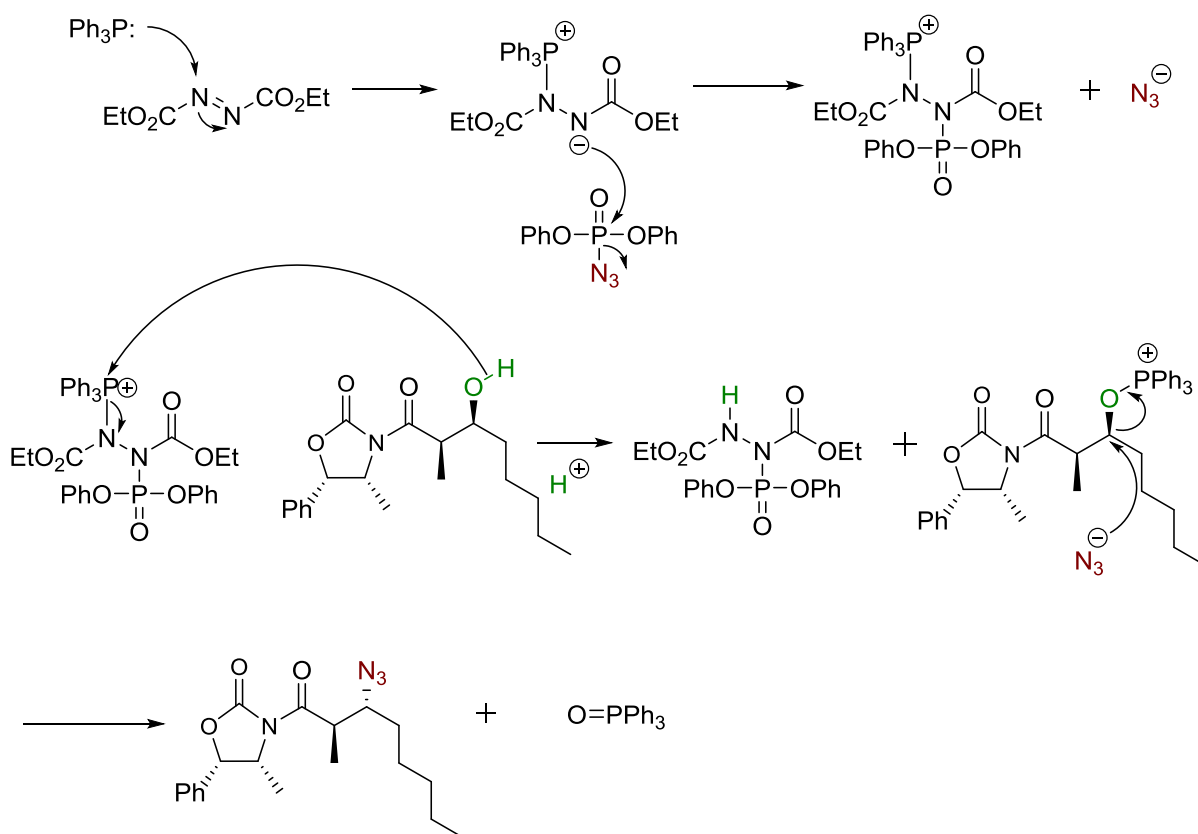
Next, the phosphorus species from step b interacts with the alcohol component. Oxygen and phosphorus have a strong mutual affinity, therefore they form the key oxyphosphonium ion, as shown in Scheme 34 step c.



Finally, the attack of the nucleophile anion upon the oxyphosphonium intermediate generated in step c gives the desired product and the triphenylphosphine oxide, as shown Scheme 34 in step d.

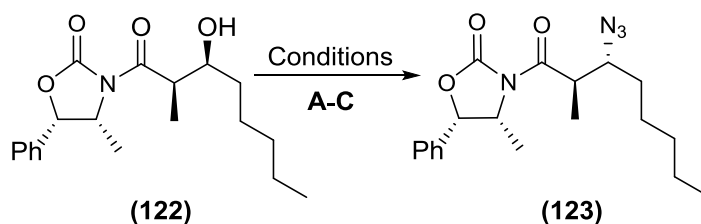
**Scheme 34.** Mitsunobu reaction mechanism.

A possible mechanism for the conversion of the aldol (**122**) to azide (**123**), by Mitsunobu reaction with diphenylphosphoryl azide (DPPA) is shown below:



**Scheme 35.** Mechanism of the Mitsunobu reaction occurring on (**122**) with DPPA.

Thompson *et al.*<sup>186</sup> were the first to report the Mitsunobu reaction using DPPA as a source of azide. In this reaction, formation of an activated oxyphosphonium ion intermediate involves liberation of azide ion from DPPA, which is then available for subsequent  $S_N2$  reaction. (see Scheme 35).



**A:**  $\text{Ph}_3\text{P}$ , DEAD, DPPA,  $\text{CH}_2\text{Cl}_2$ , r.t. 16 h; **B:** as **A**,  $0\text{ }^\circ\text{C}$  to r.t. 16 h; **C:** as **A**, using THF, 16 h.

**Scheme 36.** Mitsunobu reactions with (**122**) and DPPA as azide source.

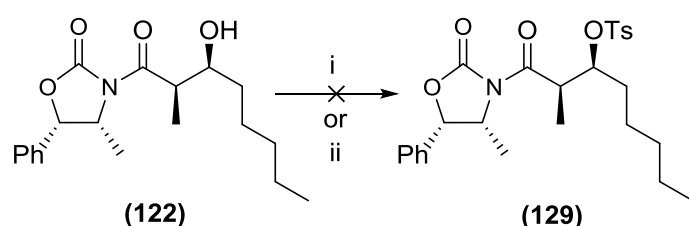
Having successfully reproduced the Crimmins syn aldol product (**122**), in the next step the introduction of a nitrogen function was attempted through Mitsunobu reaction. Three sets of

conditions were attempted as shown in Scheme 36. Firstly (**122**) was treated with DEAD, PPh<sub>3</sub> and DPPA in CH<sub>2</sub>Cl<sub>2</sub> at room temperature for 16 h. This gave the desired azide (**123**) in only 7% yield and the unchanged starting material (**122**) in 93% yield, after purification by column chromatography. In the next attempt, the reaction was repeated with PPh<sub>3</sub> and DEAD added at 0 °C, followed by addition of DPPA, then the reaction was warmed up to room temperature for 16 h. This again gave the azide (**123**) in very low yield (6%), together with the unchanged starting material (**122**) in 94% yield. The reaction was again repeated with all the reagents added at room temperature but using anhydrous THF as a solvent. This reaction was unsuccessful and gave only the unchanged starting material (**123**).

The <sup>1</sup>H NMR spectrum obtained for (**123**) was consistent with the introduction of the azide function at C-2 with inversion of configuration and a resulting anti relationship for H-2 and H-3. This assignment was based on the coupling constant  $J_{H2-H3} = 9.3$  Hz associated with the H-2 signal at  $\delta = 3.86$ . This is consistent with the value reported by Kimura *et al.*,<sup>173</sup> in the analogous conversion of a syn (2*R*, 3*S*) aldol to the anti (2*R*, 3*R*) azide, *via* S<sub>N</sub>2 displacement of an intermediate mesylate (see Scheme 38). The intermediate tosylate with syn configuration shows a correspondingly smaller  $J_{H2-H3}$ .

#### 5.2.6. Synthesis of (**123**) *via* a tosylate

Due to the lack of success in the production of the azido derivative (**123**) *via* Mitsunobu reaction, an alternative strategy was explored. It was decided to activate the hydroxy of (**122**) followed by nucleophilic displacement with azide. To achieve this, formation of the tosylate from (**122**) was attempted.



*Reagents and conditions:* (i) TsCl, pyridine, 0 °C, 3 h; (ii) TsCl, pyridine, 0 °C to 50 °C, 16 h.

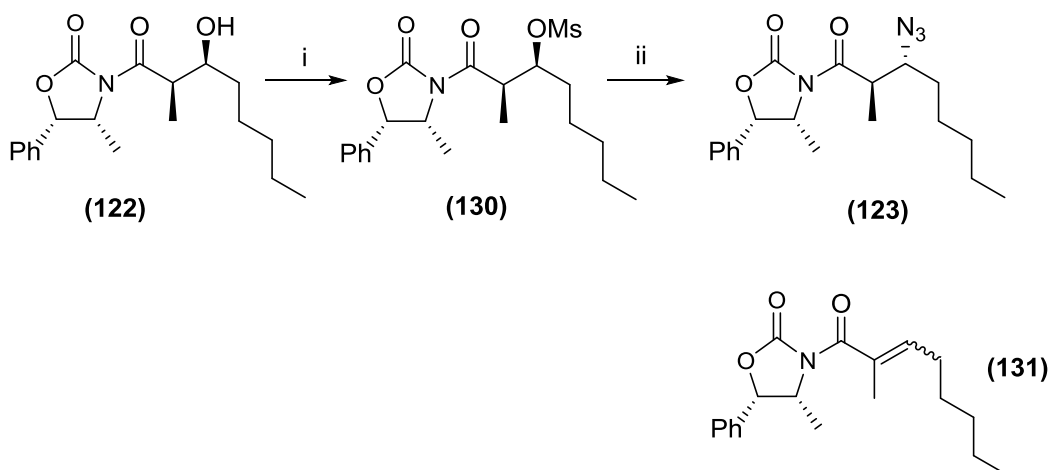
**Scheme 37.** Attempted conversion of aldol (**123**) to tosylate (**129**).

In the first attempt, (**122**) was treated with tosyl chloride at 0 °C in anhydrous pyridine then warmed up to room temperature. The reaction was monitored by TLC, which showed a new higher running material, although after work up of the reaction, the <sup>1</sup>H NMR spectrum of the

crude product indicated unchanged starting material (**122**). The reaction was repeated under the same conditions as before but after adding all the reagents at 0 °C, the reaction mixture was heated at 50 °C for 16 h. The appearance of the TLC was similar to before and the  $^1\text{H}$  NMR spectrum of the crude product again indicated unchanged starting material (**122**), as outlined in Scheme 37.

### 5.2.7 Synthesis of (**122**) via the mesylate

In the next attempt, the hydroxy group from (**122**) was activated as a mesylate. For this (**122**) was treated with  $\text{Et}_3\text{N}$  and methanesulfonyl chloride to give the corresponding mesylate (**130**), as confirmed by the  $^1\text{H}$  NMR spectrum of the crude product. (**130**) was treated with  $\text{NaN}_3$  at 70 °C for 3 h and this gave the desired azide (**123**) in 27% yield plus the eliminated material (**131**) in 42% yield. The reaction was repeated under the same conditions as before but at a lower temperature, 40 °C, and it was monitored by TLC. After 15 min reaction TLC showed a new higher running material. In this case, after work up of the reaction, the  $^1\text{H}$  NMR spectrum of the crude product revealed no (**123**) but only the eliminated product (**131**), which was isolated in 18% yield material plus the unchanged starting material in 39% yield, as shown in Scheme 38.



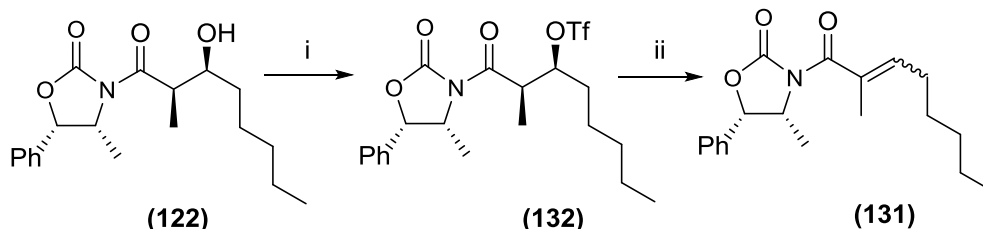
*Reagents and conditions:* (i)  $\text{MeSO}_2\text{Cl}$ ,  $\text{Et}_3\text{N}$ ,  $\text{CH}_2\text{Cl}_2$ , 0 °C, 3 h; (ii)  $\text{NaN}_3$ , 15-crown-5, DMF, 70 °C, 3 h.

**Scheme 38.** Preparation and attempted displacement of mesylate (**130**).

### 5.2.8. Synthesis of (**123**) via the triflate

A solution of (**122**) was treated with pyridine and triflic anhydride in  $\text{CH}_2\text{Cl}_2$ , to give the corresponding triflate derivative (**132**) in 62% yield, confirmed by the  $^1\text{H}$  NMR spectrum of the

crude product. The nucleophilic displacement was attempted with  $\text{NaN}_3$  at 60 °C in DMF for 16 h. Analysis by  $^1\text{H}$  NMR showed only elimination products (**131**) alongside some other unidentified materials (Scheme 39).



*Reagents and conditions:* (i)  $\text{OTf}$ , pyridine,  $\text{CH}_2\text{Cl}_2$ , 0 °C, 2 h 30 min; (ii)  $\text{NaN}_3$ , DMF, 60 °C, 16 h.

**Scheme 39.** Synthesis and attempted displacement of triflate (**132**).

### 5.2.9. Conclusion and future work on aldol reactions

A potential approach to the novel amino acid (**105**) and its diastereoisomers has been studied based on the Crimmins modification of the Evans aldol reaction. Aldol intermediates (**113**) and (**122**) were both successfully prepared following Crimmins titanium enolate conditions and the syn stereochemistry of the two products was deduced primarily based on the value of the  $J_{\text{H}2-\text{H}3}$  coupling constants observed in the  $^1\text{H}$  NMR spectra obtained and by comparison of the NMR data with those reported for the same compounds prepared by Evans boron enolate chemistry.

Although aldol derivatives (**122**) and (**113**) were successfully prepared, introduction of the nitrogen function by nucleophilic displacement proved to be problematic, leading to eliminated products. It was therefore decided to pursue an alternative approach which would involve introducing the C-3 methyl group into an enantiomerically pure  $\alpha$ -amino derivative.

## 5.3. Chiral pool approach towards the synthesis of Amoa

### 5.3.1. Summary on the aldol strategy

Our first strategy depended upon the specific and high-yielding Crimmins modification of the Evans aldol reaction.<sup>176</sup> We successfully obtained syn adducts by this method as single diastereoisomers. The initial route involved the introduction of the nitrogen function at the beginning of the synthesis *via* an inversion procedure on an aldol product (**122**) through a Mitsunobu reaction,<sup>187</sup> which gave poor results. Subsequently we focused on alternative routes *via* nucleophilic substitution reactions of a mesylates, tosylate, or triflate. The best conditions for introducing an azido function into (**122**) were *via* the mesylate (**130**). However, under optimized conditions, significant amounts of eliminated material were obtained along with the desired product (**123**). It was therefore decided that it would not be useful to attempt the same strategy with aldol compound (**113**), which has an  $\alpha$ -substituent of similar steric bulk to (**122**).

Because of the limited success in introducing the nitrogen function of the desired amino acid *via* displacement reactions, it was decided to explore an alternative approach using a chiral starting material with the key nitrogen function already in place.

### 5.3.2. Aspartic and glutamic acid starting scaffolds

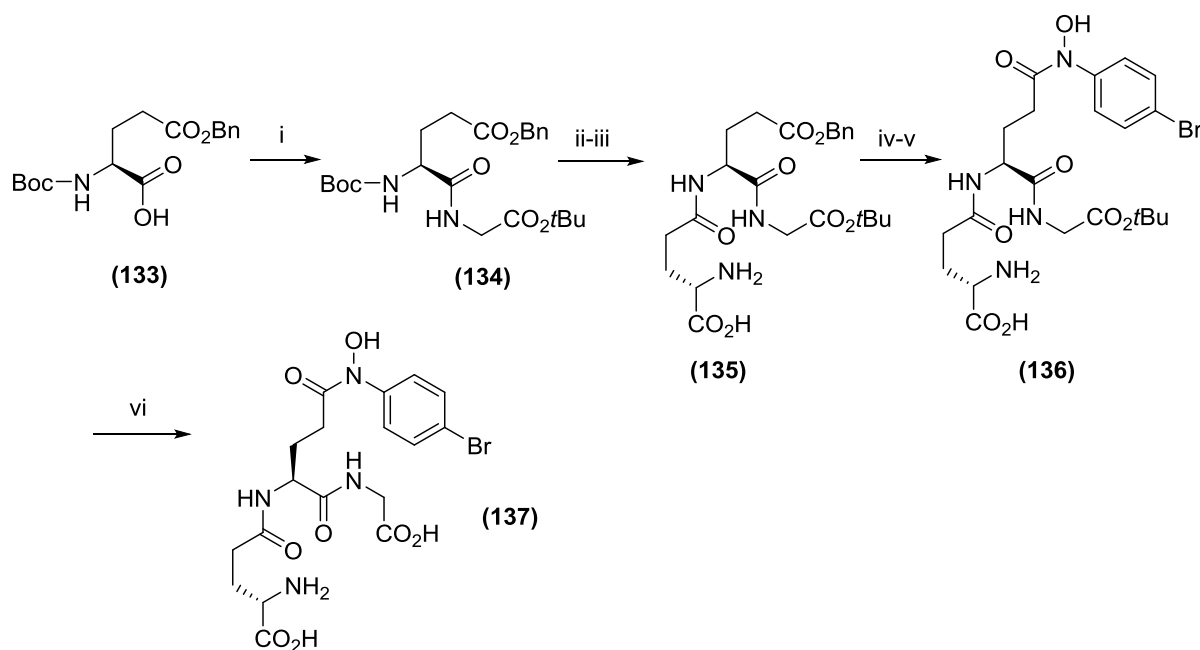
Aspartic and glutamic acids play important roles as general acids in enzyme active centres,<sup>188</sup> as well as in maintaining the solubility and ionic character of proteins. They have also been used extensively as enantiomerically pure starting materials or building blocks for the preparation of natural products or novel biologically active molecules.

The purpose of using aspartic and glutamic acids as starting materials in organic synthesis is to introduce one or more defined stereogenic centres within a biological active target. The possibility to generate a  $\beta$ - or  $\gamma$ -enolate in the side chain provides many possibilities to develop more complex structures, starting with a fixed configuration at the  $\alpha$ -carbon, according to whether D- or L- starting materials are chosen. The  $\beta$ - or  $\gamma$ -carboxylic acid function also provides a way to simply attach novel side chain groups. A few examples are outlined below.

The *N*-aryl-*N*-hydroxycarbamoyl derivative (**137**) is a competitive inhibitor of glyoxalase I, which is involved in the prevention of cell apoptosis in tumours.<sup>189</sup> The molecule is an analogue of glutathione ( $\gamma$ -Glu-Cys-Gly-OH) in which a Glu residue is used to replace cysteine. The carboxylic acid function is then converted to a hydroxamide, which is able to chelate  $\text{Zn}^{2+}$ .



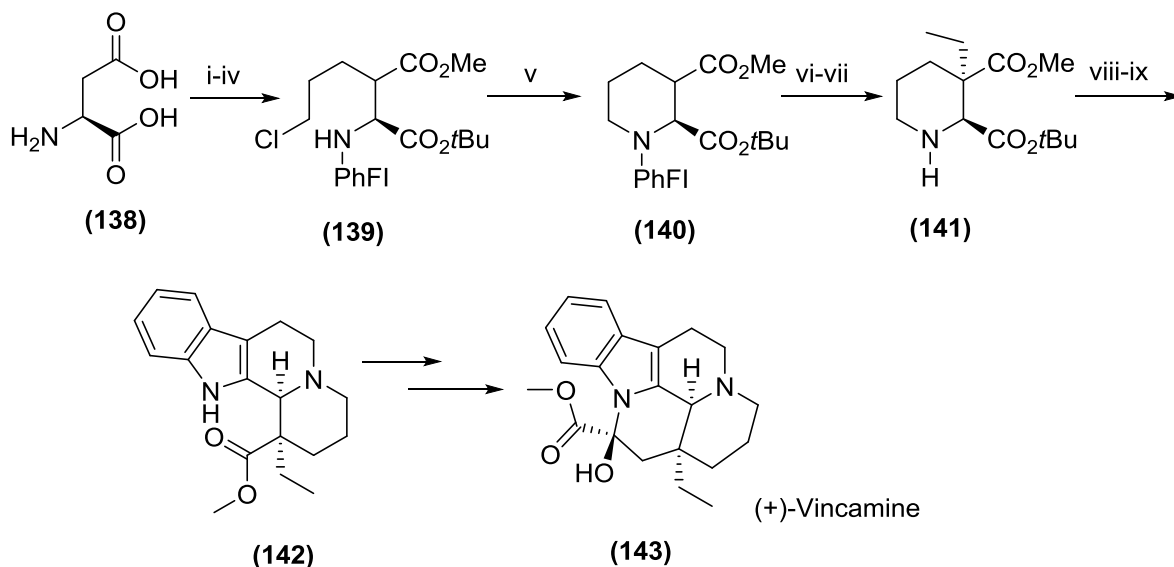
More and Vince<sup>189</sup> have reported the synthesis of the tripeptide Glu-Glu-Gly, which began with coupling of Boc-Glu(OBn)-OH (**133**) with H-Gly-OtBu to give the dipeptide (**134**). The Boc group was selectively cleaved, then the resulting dipeptide was coupled to Boc-Glu-OtBu to give the tripeptide. Finally the hydrogenolysis of the side-chain benzyl ester of the tripeptide provided acid (**135**). To complete the synthesis, *p*-bromophenylhydroxylamine was coupled to the acid (**135**) using DCC and DMAP to give (**136**), followed by deprotection of the acid-labile groups to give (**137**), as outlined in Scheme 40.



*Reagents and conditions:* (i) HCl, H-Gly-OtBu, EDC, HOBt, NMM, CH<sub>2</sub>Cl<sub>2</sub>, 91%; (ii) 4M HCl/dioxane, 0 °C, 99%; (iii) Boc-Glu-OtBu, EDC, HOBt, CH<sub>2</sub>Cl<sub>2</sub>, 84%; (iv) H<sub>2</sub>, Pd/C, MeOH, 86%; (v) *p*-bromophenylhydroxylamine, EDC, CH<sub>2</sub>Cl<sub>2</sub>, 52%; (vi) TFA, CH<sub>2</sub>Cl<sub>2</sub> (1:1), 76%.

**Scheme 40.** Synthesis of an S-(*N*-aryl-*N*-hydroxamate) analogue of glutathione.<sup>189</sup>

### 5.3.3. Aspartic acid as a scaffold in alkaloid synthesis



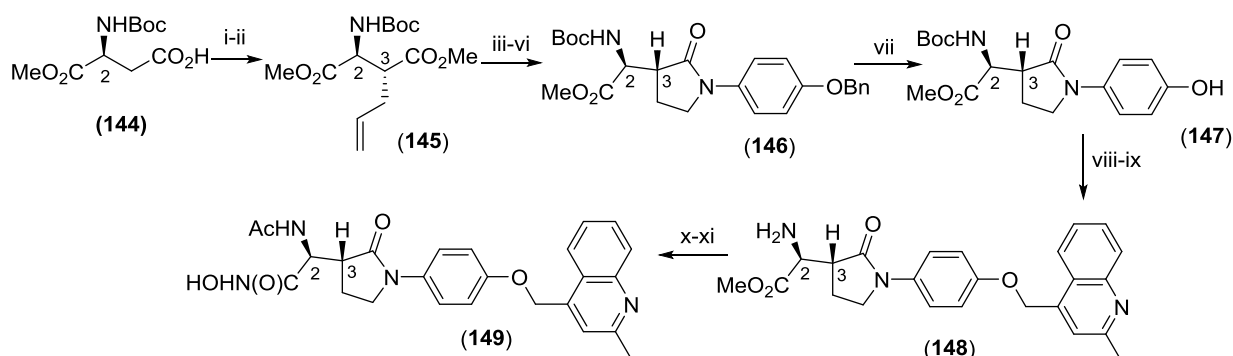
*Reagents and conditions:* (i) acetyl chloride, MeOH, 8 °C 30 min, then rt, 15 h, 77%; (ii) TMSCl, CHCl<sub>3</sub>, rt, 2 h, then Et<sub>3</sub>N, Pb(NO<sub>3</sub>)<sub>2</sub>, 9-bromo-9-phenylfluorene 3 d, 92%; (iii) *O*-*tert*-butyl-*N,N'*-diisopropylisourea, CH<sub>2</sub>Cl<sub>2</sub>, rt, 44 h, then H<sub>2</sub>O, 1 h, 84%; (iv) KHMDS in toluene, THF, -78 °C 45 min, then 3-chloropropyl triflate, 1 h 30 min, 76%; (v) NaHCO<sub>3</sub>, CH<sub>3</sub>CN, reflux 10 min, then NaI, 48 h, 81%; (vi) DIPEA, *n*-BuLi in hexanes, THF, -78 °C, 40 min, then ethyl iodide, 2 h 30 min, 59%; (vii) 10% Pd/C, AcOH, H<sub>2</sub>, rt, 2 h 30 min, 98%; (viii) tryptophyl bromide, NaHCO<sub>3</sub>, CH<sub>3</sub>CN, 70 °C 21 h, 93%; (ix) *i*PrOH/H<sub>2</sub>O (v/v), AcOH, 100 °C 15 h, then PhPOCl<sub>2</sub>, 15 min, 74%.

**Scheme 41.** Synthetic route to (+)-vincamine, based on L-Asp diesters.<sup>190</sup>

The synthetic route to (+)-vincamine, which is an alkaloid possessing cerebral vasodilatory effects, is outlined in Scheme 41.<sup>190</sup> An aspartate derivative with different protecting groups at the  $\alpha$ - and  $\beta$ - carboxyl functions was first mono-C-alkylated on the enolate of the  $\beta$ -ester with the three-carbon bis-electrophile, 3-chloropropyl trifluoromethanesulfonate to give (139), as a mixture of diastereoisomers. This was followed by an intramolecular cyclisation to give a piperidine derivative (140). A second alkylation of the ester enolate was then used to introduce the ethyl group in (140). The PhFI group was removed to give (141) and then *N*-alkylation with tryptophyl bromide was carried out. The synthesis of the key tetracyclic intermediate (142) was then finally accomplished by cyclisation of an intermediate iminium ion generated after the hydrolysis and decarboxylation at the  $\alpha$ -*tert*-butyl ester.

### 5.3.4. Aspartic acid for formation of lactams

Aspartic acid has been used as the starting material to synthesize lactam (**149**), which is an inhibitor of tumour necrosis factor- $\alpha$  converting enzyme (TACE).<sup>191</sup> Boc-Asp-OMe (**144**) was first esterified with MeI and DBU to give the diester. This was then alkylated with allyl bromide to give anti-product (**145**). After ozonolysis, the resultant aldehyde formed a Schiff base with 4-benzyloxylaniline, followed by reduction of the imine by NaBH(OAc)<sub>3</sub> and cyclisation in toluene at reflux to provide lactam (**146**). After removal of the benzyl ether protection, the 2-methyl-4-quinolinyl)methyl group was incorporated, followed by Boc deprotection to give the free amine (**148**). To complete the synthesis, acetylation and hydroxamic acid formation were carried out on the free amine and on the C- terminal carboxylic acid, respectively to give (**149**). Here the Asp starting material fixes the C-2 stereocentre and also allows the generation of the C-3 stereocentre.



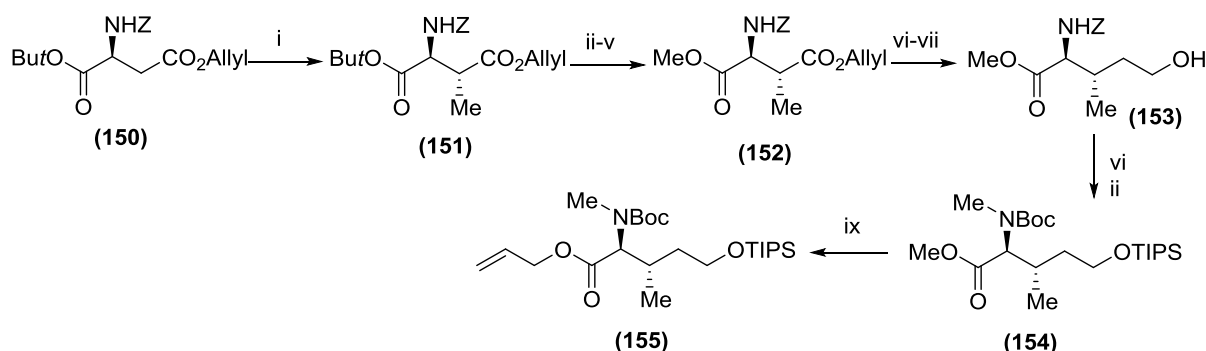
**Reagents and conditions:** (i) MeI, DBU, toluene, at reflux, 96%; (ii) allyl bromide, LiHMDS, toluene, THF, 61%, (iii) O<sub>3</sub>, CH<sub>2</sub>Cl<sub>2</sub>, PPh<sub>3</sub>, 71%; (iv) 4-benzyloxylaniline, toluene, at reflux; (v) NaBH(OAc)<sub>3</sub>, ClCH<sub>2</sub>CH<sub>2</sub>Cl; (vi) toluene at reflux 46% for 3 steps; (vii) H<sub>2</sub>, Pd(OH)<sub>2</sub>/C, MeOH, 74%; (viii) 4-chloromethyl-2-methylquinoline, Cs<sub>2</sub>CO<sub>3</sub>, DMSO, 86%; (ix) CF<sub>3</sub>CO<sub>2</sub>H, CH<sub>2</sub>Cl<sub>2</sub>, quantitative yield; (x) Ac<sub>2</sub>O, i-Pr<sub>2</sub>NEt, CH<sub>2</sub>Cl<sub>2</sub>, 87%; (xi) NH<sub>2</sub>OH, KOH, MeOH, 51%.

**Scheme 42.** Preparation of a lactam inhibitor (**149**) of tumour necrosis factor- $\alpha$  converting enzyme from L-Asp.<sup>191</sup>

### 5.3.5. Aspartic acid in the synthesis of the novel amino acid NMeOHile

The *N*-methyl amino acid *N*-methyl- $\delta$ -hydroxyisoleucine (NMeOHile) is found in haliptin A, a peptide which has a potent anti-inflammatory activity.<sup>192</sup> The synthetic route presented by Yu *et al.*,<sup>193</sup> was based on a highly diastereoselective methylation of a selectively protected

aspartate, Z-Asp(OAll)-OtBu, followed by building up the molecule to the desired NMeOHlle (see Scheme 43 ).



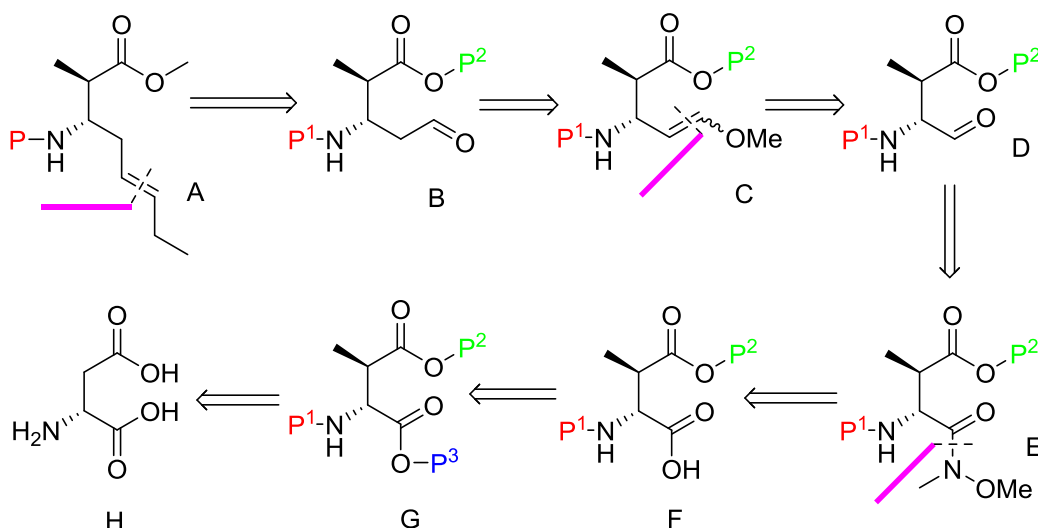
*Reagents and conditions:* (i) LiHMDS, THF, HMPA then MeI (ii) TFA, CH<sub>2</sub>Cl<sub>2</sub>, then CH<sub>2</sub>N<sub>2</sub>, Et<sub>2</sub>O, rt, 95%; (iii) [Pd(PPh<sub>3</sub>)], NMA, CH<sub>2</sub>Cl<sub>2</sub>, rt; (iv) ClCOiBu, NMM, THF, -20 °C, then CH<sub>2</sub>N<sub>2</sub>, Et<sub>2</sub>O, 20 °C to rt; (v) AgNO<sub>3</sub>, H<sub>2</sub>O, THF; (vi) ClCO<sub>2</sub>Et, NMM, THF, 0 °C then NaBH<sub>4</sub>, MeOH, 0 °C to rt, 35% yield for the 4 steps; (vii) TIPSCl, imidazole, DMAP, CH<sub>2</sub>Cl<sub>2</sub>, rt, 89%; (viii) Pd/C, H<sub>2</sub>, Boc<sub>2</sub>O, MeOH, rt; (ix) Ag<sub>2</sub>O, MeI, DMF, 50 °C, 94% yield for 2 steps; (x) aq. LiOH, THF, MeOH, then allyl bromide, K<sub>2</sub>CO<sub>3</sub>, DMSO, rt, 92%.

**Scheme 43.** Synthesis of NMeOHlle from L-Asp.<sup>193</sup>

Here Asp as a starting material allows the β-methyl group to be introduced into the amino acid structure with control of stereochemical configuration.

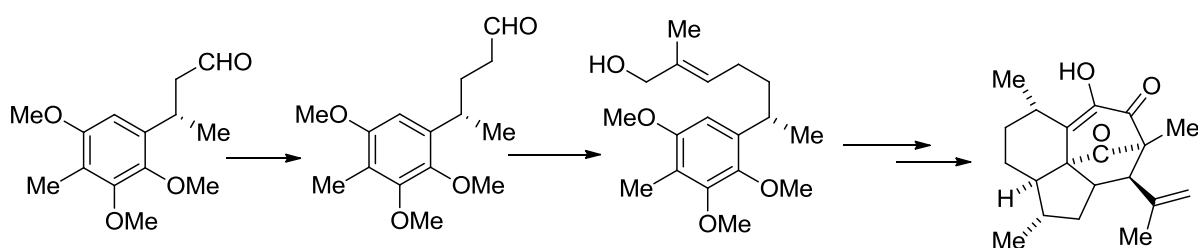
### 5.3.6. Aspartic acid used in our strategy towards the synthesis of Amoa

The approach of Yu *et al.*,<sup>193</sup> and similar work suggested to us that this might be a suitable strategy for the preparation of Amoa. This seemed promising for, if L-aspartic acid was chosen as the chiral educt for (119), its nitrogen function, which was problematic to introduce and also one of the stereogenic centres necessary for the future (105), would already be in place. The β-carboxyl group should allow introduction of an alkyl group *via* the β-ester enolate and this should lead to the formation of the second asymmetric carbon, required in (119). The α-carboxyl group could then be derivatized to an aldehyde function which could be elongated to the Amoa side chain.



**Scheme 44.** Planned retrosynthesis of compound (**119**).

The retrosynthetic analysis of (**119**) is described in Scheme 44. The key steps are the stereoselective introduction of the side-chain methyl group and the homologation of an  $\alpha$ -aminoaldehyde derivative to a  $\beta$ -aldehyde. We envisaged that a suitable aldehyde could be prepared by chemoselective reduction of a Weinreb amide<sup>194</sup> or similar Asp derivative.<sup>195</sup> For the conversion of an  $\alpha$ -aldehyde into the  $\beta$ -aldehyde, we anticipated using a Wittig reaction with (methoxymethylene)triphenylphosphorane to generate an enol ether, followed by acidic hydrolysis, to give the  $\beta$ -aldehyde. This approach has been applied by Ying *et al.*,<sup>196</sup> for the synthesis of the antitubercular agent elisapterosin B, in which the key intermediate, a *cis* alkene, undergoes diastereoselective ring closure using mercury salts, as shown in Scheme 45.

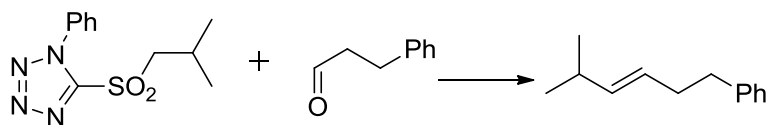


*Reagents and conditions:* MeOHC=PPh<sub>3</sub>, acetone, water, *p*-toluenesulfonic acid, 15 min, rt.

**Scheme 45.** Wittig reaction, using an enol ylide for aldehyde homologation.<sup>196</sup>

In our synthesis, the alkene function could be introduced into the molecule by a modified Julia-Kocienski reaction. Recently Pospíšil has developed new conditions for the Julia-Kocienski

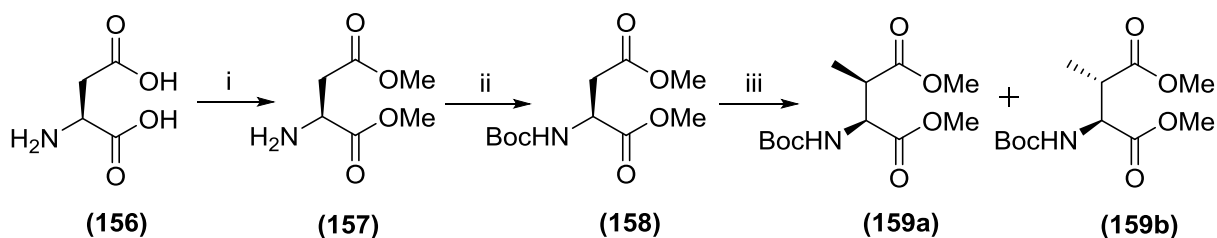
olefination, which uses specific metal cation-chelating agents to enhance the *E*-selectivity, as shown in Scheme 46.<sup>197</sup>



*Reagents and conditions:* KHMDS, 18-crown-6, THF, -78 °C.

**Scheme 46.** Modified Julia-Kocienski olefination by Pospíšil.<sup>197</sup>

Our initially adopted approach *via* (**159a**) is outlined in Scheme 47. The advantage of this synthesis is that starts with a readily available Asp diester, easily produced in large quantities. Park *et al.*,<sup>198</sup> reported that the alkylation of (**158**) gave a 1:1 mixture of diastereoisomers but this was considered not to be necessarily a problem. In our case if we could separate both isomers (**159a**) and (**159b**) by column chromatography, this would provide ultimately a route to the synthesis of all four stereoisomers of Amoa, starting from L- or D-aspartic acid, using the approach in Scheme 47.



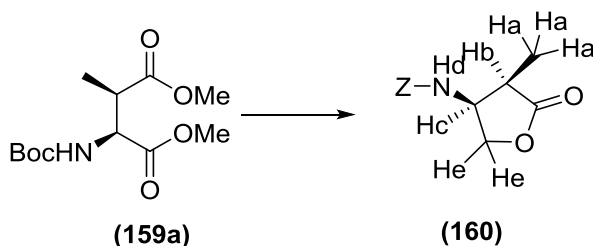
*Reagents and conditions:* (i) MeOH, TMSCl, 0 °C, then warm up to rt, 48 h; (ii) Boc<sub>2</sub>O, Et<sub>3</sub>N, MeOH, 0 °C, then warm up to rt, 16 h; (iii) MeI, LiHMDS, THF, -78 °C, 6 h, then warm up to rt, 16 h.

**Scheme 47.** Synthesis of the  $\beta$ -methyl-L-Asp derivatives (**159a**) and (**159b**).<sup>198</sup>

Our first synthesis of (**159a**) began by dissolving commercial L-aspartic acid (**1**) in MeOH and treating with TMSCl. After 48 h reaction, all volatiles were removed *in vacuo* and the crude diester salt (**157**) was directly *N*-acylated using Boc anhydride in the presence of triethylamine to give (**158**) in 53% yield. The enolate dianion that was obtained by treating (**158**) with 2 eq of lithium hexamethyldisilazane (LiHMDS) at -78 °C was then allowed to react with iodomethane to give (**159a**) in 17% yield as a *single* isomer isolated by column chromatography. The methylation gave a mixture of diastereoisomers in a ratio of 3:1 (**159a**:

**159b**), based on the  $^1\text{H}$ NMR spectrum of the crude product. The overall isolated yield of (**159a**) and (**159b**) was 49% , consisting of pure (**159a**) in 17% yield, a 1:1 mixture of (**159a**) and (**159b**) in 25% yield and (**159b**) in 7% yield.

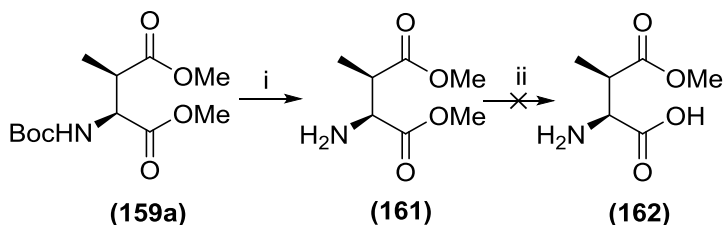
The NMR spectrum of (**159a**) obtained was identical to that reported by Park *et al.*<sup>198</sup> In this study, the (2*S*, 3*R*) configuration of the alkylation product was confirmed by conversion of (**159a**) to the lactone (**160**). The stereochemical configuration was confirmed by  $^1\text{H}$  NMR, with strong NOEs being observed between  $\text{H}_\text{a}$ - $\text{H}_\text{d}$  and  $\text{H}_\text{b}$ - $\text{H}_\text{c}$  that confirms this assignment (Scheme 48).



**Scheme 48.** Proof of the stereochemical configuration of (**159a**).<sup>198</sup>

The ratio of isomers was calculated based on the relative integrals of the  $\text{H}_\beta$  protons for the two isomers in the  $^1\text{H}$  NMR crude mixture. The preference for the *anti* isomer (**159a**) is in contrast to Park's report<sup>198</sup> but consistent with other studies.<sup>199</sup> Park *et al.*,<sup>198</sup> reported (**159a**) and (**159b**) as a 1:1 mixture of diastereoisomers, which were separable by column chromatography, (see above).

With (**159a**) from the first key transformation in hand, the next step was the selective removal of the  $\alpha$ -ester, in order to carry out the proposed homologation. This involved first removal of the Boc group in acidic conditions, then treatment with basic copper carbonate.

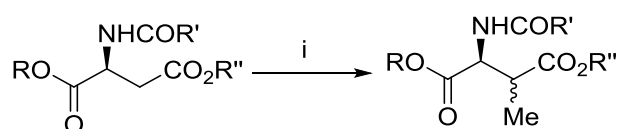


*Reagents and conditions:* (i) 4 M aq. HCl 30 min, rt; (ii)  $\text{CuCO}_3 \cdot \text{Cu}(\text{OH})_2$ , thioacetamide, 70 °C, 3 h

**Scheme 49.** Attempted  $\alpha$ -ester hydrolysis (**159a**).<sup>200</sup>

The selective hydrolysis of dimethyl L-aspartate hydrochloride has been reported by Gmeiner *et al.*<sup>190</sup> but this method uses hydrogen sulfide to remove the copper salts at the end of the reaction. In our case when the selective hydrolysis was attempted with work-up with thioacetamide,<sup>200</sup> the desired compound (**162**) could not be isolated, as shown in Scheme 49.

Although the alkylation reaction was successfully achieved, the differentiation of the  $\alpha$ - and  $\beta$ -carboxylic acid functions was an issue and therefore we decided to change the protecting groups. It is known that the diastereoselectivity in  $\beta$ -alkylation reactions of Asp acid derivatives depends on the protecting groups on the  $\alpha$ - and  $\beta$ -acids and nitrogen functions,<sup>199, 201</sup> as demonstrated in Table 4. The diastereoselectivities observed in our experiments with (**158**) are consistent with those reported for Boc-Asp(OBn)-OMe<sup>193</sup> (entry 2), which is the closest analogue to (**158**).



*Reagents and conditions:* (i) LiHMDS, HMPA, THF, 45 min, distilled MeI, -78 °C, 3 h.

**Scheme 50.** Alkylation of aspartic acid derivatives under Hanessian's conditions.<sup>199</sup>



Table 4. Methylation of aspartate derivatives<sup>199</sup>

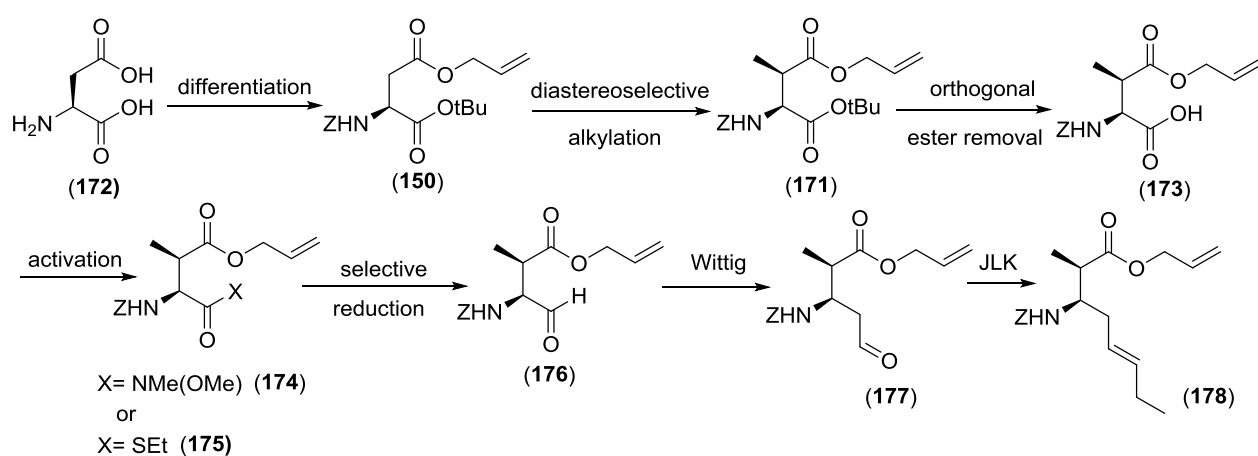
Entry	Substrate	R	R'	R''	Product	Yield %	<i>anti/syn</i>
1	<b>163</b>	Me	Bn	TMSE	<b>164</b>	81	1.5:1
2	<b>165</b>	Me	<i>t</i> Bu	Bn	<b>166</b>	74	2.3:1
3	<b>167</b>	<i>t</i> Bu	Bn	TMSE	<b>168</b>	77	3.5:1
4	<b>169</b>	<i>t</i> Bu	Bn	Me	<b>170</b>	94	>20:1
5	<b>150</b>	<i>t</i> Bu	Bn	allyl	<b>171</b>	91	>20:1

Typical procedure: A stirred solution of LiHMDS (39 mL, 39 mmol) and HMPA (20 mL), was treated with a solution of Asp derivative (6.72 g, 18.6 mmol) in dry THF (80 mL), added dropwise. After 45 min, freshly distilled MeI (3.5 mL, 56.2 mmol) was added to the reaction mixture at -78 °C and stirring continued for 3 h.

Yu *et al.*,<sup>193</sup> reported the methylation of protected aspartate derivatives under Hanessian's reaction conditions,<sup>199</sup> using the strong base, LiHMDS, and a mixture of solvents, THF/HMPA and LiHMDS, as outlined in Table 4. The alkylation reaction showed poor diastereoselectivity when bulky protecting groups on the  $\beta$ -carboxylic acid were used, this applied for (**163**), (**165**) and (**167**). In a different study Humphrey *et al.*,<sup>202</sup> demonstrated that bulkier *N*-protecting groups favour the *anti* selectivity and this rule seems to fulfil the model proposed by Yu *et al.*;<sup>193</sup> when  $\alpha$ -*tert*-butyl ester (**167**) was used, the ratio for *anti* and *syn* isomers was then enhanced to 3.5:1. Excellent diastereoselectivity could be achieved by reducing the size of the  $\beta$ -ester moiety (entries **4** et **5**). The conclusion was, therefore, that, in the methylation reaction, larger  $\alpha$ - and small  $\beta$ -esters in the aspartate are favoured for *anti* selectivity. However, although compound (**169**) gave higher *anti* selectivity, the hydrolysis of the alkylated product (**170**) with aqueous LiOH in methanol gave a mixture of the acid plus the 3-epimer. For this reason, Yu *et al.*,<sup>193</sup> preferred the allyl ester derivative (**150**).

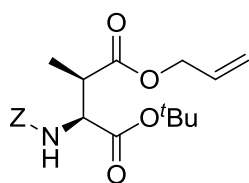
Our particular attention was attracted to (**150**), this *N*-benzyloxycarbonyl derivative should favour *anti* selectivity, as shown in entry 5 on Table 4.<sup>199</sup> We therefore decided to use (**171**) for our synthetic approach, with its particular combination of protecting groups that should give mainly one stereoisomer, to allow us to put our planned Scheme 51 into action. The  $\alpha$ - and the

$\beta$ -carboxylic acid groups are also selectively protected which should allow us to build selectively both sides of the molecule.



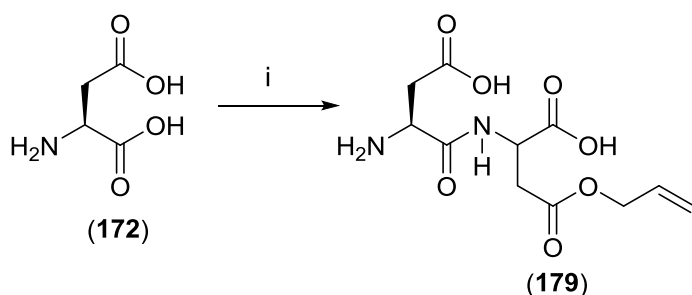
**Scheme 51.** Planned synthesis for Amoa from L-aspartic acid.

### Preparation of (2S)-4-allyl-1-tert-butyl-2-(((benzyloxy)carbonyl)amino)succinate (150)



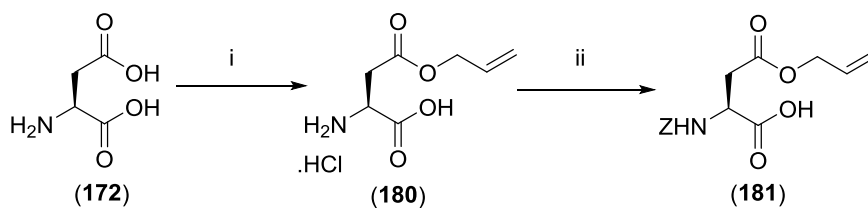
**Figure 36.** Compound (150)

The success of this strategy hinged on the availability of an allyl ester derivative. Allyl ester groups can be removed by the use of tetrakis(triphenylphosphine)palladium(0) and so can be removed independently of benzyloxycarboxyl groups which are removable by catalytic hydrogenolysis and *tert*-butyl groups which are acid-labile.



Method A. *Reagents and conditions* : (i) Allyl alcohol, TMSCl, 0 °C-rt, 18 h, 53%.

**Scheme 52.** Attempted preparation of (180) – Method A.<sup>203</sup>



*Reagents and conditions:* (i) Allyl alcohol, acetyl chloride, 0 °C-rt, 83%; (ii) Z-Cl, K<sub>2</sub>CO<sub>3</sub>, H<sub>2</sub>O, rt, 16 h, 56%.

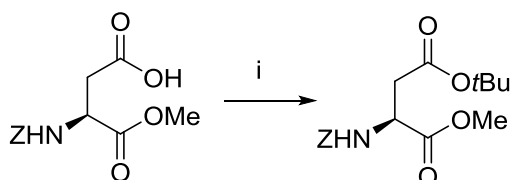
**Scheme 53.** Preparation of Z-Asp(OAll)-OH - Method B.<sup>204</sup>

The preparation of the H-Asp(OAll)-OH, (**180**) was attempted by two methods. The first method (A) according to the procedure of Lajoie,<sup>203</sup> involved treatment with chlorotrimethylsilane in allyl alcohol; this appeared to give the dipeptide derivative (**179**), according to <sup>1</sup>H NMR analysis, as shown in Scheme 52. In the second method (B),<sup>204</sup> L-Asp was esterified by treatment with acetyl chloride in allyl alcohol to give (**180**) in 83% yield. The amino group of (**180**) was then N-protected, with benzyloxycarbonyl, using benzyl chloroformate, under basic conditions to give Z-Asp(OAll)-OH (**181**) in 56% yield, as shown in Scheme 53.

As noted above, it was important to protect (**181**) at the  $\alpha$ -carboxyl group with *tert*-butyl for the methylation reaction in order to ensure the formation of a single major diastereoisomer in the alkylation reaction. For this transformation several attempted reactions were carried out. We chose to avoid the classic isobutene/H<sub>2</sub>SO<sub>4</sub> method employed by Baldwin,<sup>204</sup> as this would require the use of specialised apparatus (pressure vessel) which was not available to us.

#### *Methods using Boc anhydride*

Nuijens *et al.*<sup>205</sup> have reported the  $\beta$ -esterification of Z-Asp-OMe using Boc anhydride in MeCN/*t*BuOH. This gave the desired Asp derivative in very high yield, as shown in Scheme 54.

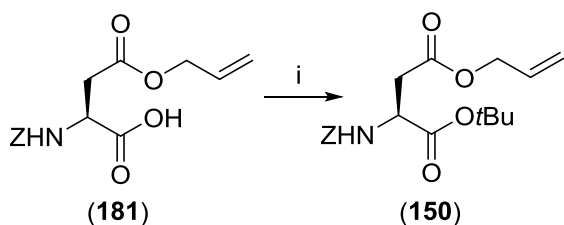


*Reagents and conditions:* (i) Boc<sub>2</sub>O, CH<sub>3</sub>CN/*t*BuOH (v/v: 4:2), pyridine, DMAP, rt, 24 h, 94%.

**Scheme 54.**  $\beta$ -esterification of Z-Asp-OMe.<sup>205</sup>

We tried to use this method for the  $\alpha$ -esterification of (**181**), as outlined in Scheme 55.

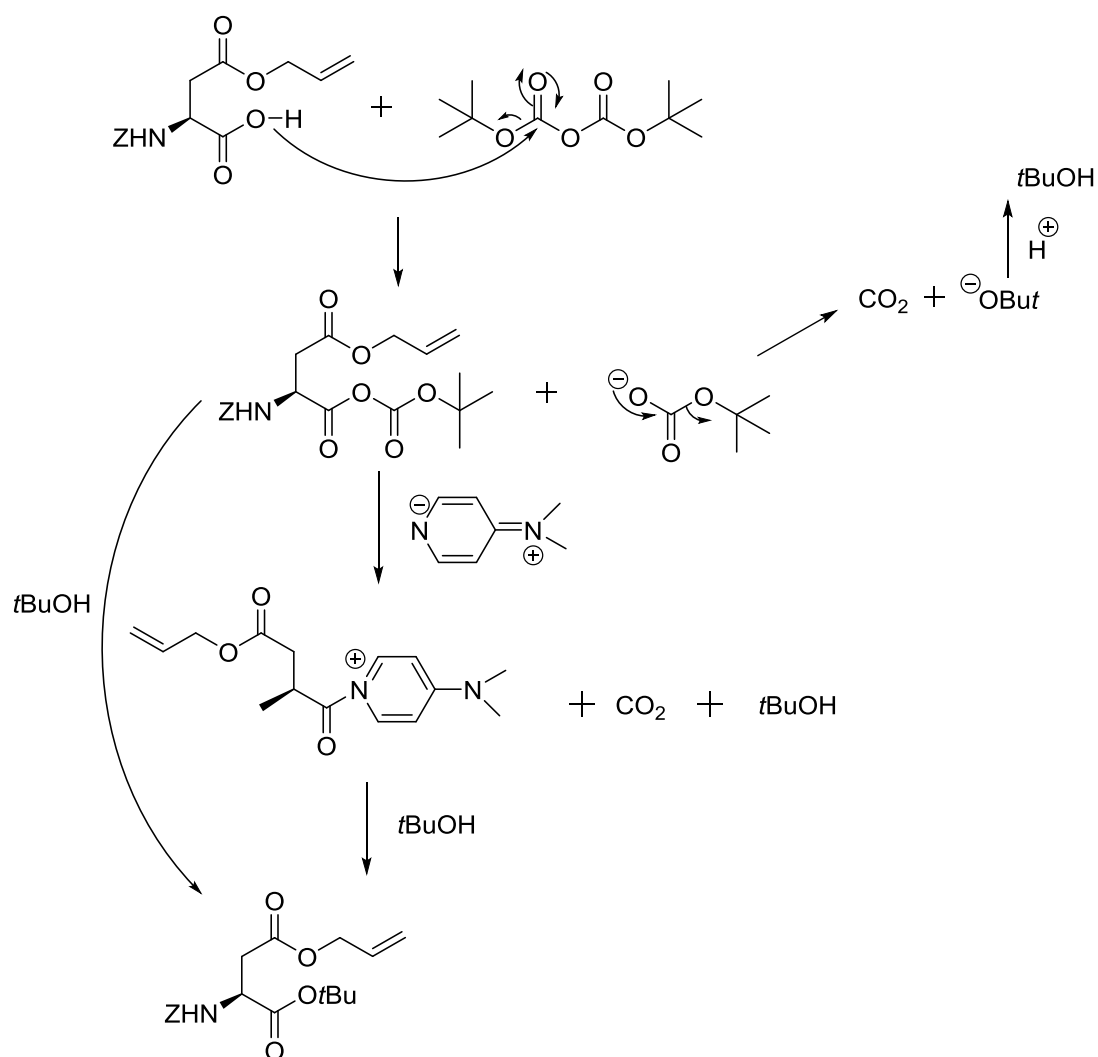
The reaction was monitored by TLC and this showed the formation of a new higher running material but a significant amount of unchanged starting material.



Method A. *Reagents and conditions*: (i)  $\text{Boc}_2\text{O}$ ,  $\text{CH}_3\text{CN}/t\text{BuOH}$  (v/v: 4:2), pyridine, DMAP, rt, 18 h, 19%.

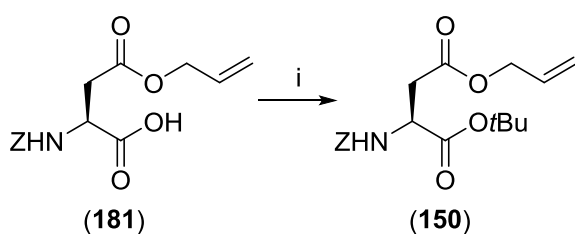
**Scheme 55.**  $\alpha$ -Esterification of (**181**).<sup>205</sup>

The expected  $\alpha$ -ester (**150**) was isolated in only in 19% yield after column chromatography. During the course of this reaction, the mixed anhydride obtained by activation with Boc anhydride of the carboxyl group liberates *tert*-butyl alcohol as a by-product. This may then itself react with the activated acid to give the corresponding ester, making thus an efficient one-pot esterification. The proposed mechanism is outlined in Scheme 56.



**Scheme 56.** Mechanism of synthesis of  $\alpha$ -aspartate esters *via*  $\text{Boc}_2\text{O}$ /DMAP.

It is known that the addition of a catalytic amount of 4-dimethylaminopyridine (DMAP) is exceedingly effective in the conversion of mixed anhydrides formed with dicarbonate reagents to the corresponding esters.<sup>206</sup>



Method B. *Reagents and conditions:* (i)  $\text{Boc}_2\text{O}$ ,  $t\text{BuOH}$ , DMAP, rt, 16 h, 24%.

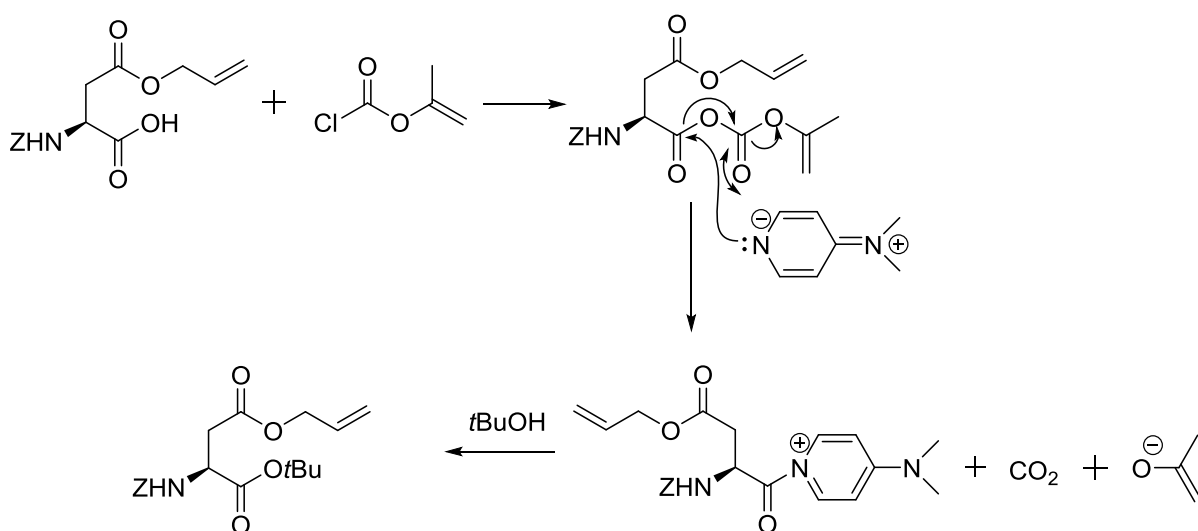
**Scheme 57.**  $\alpha$ -Esterification of Z-Asp(OAll)-OH.<sup>206</sup>

The reaction of Scheme 57 was repeated according to Takeda *et al.*,<sup>206</sup> using *t*BuOH as the only solvent but the yield was only slightly improved, giving the desired compound (**150**) in 24% yield, as shown in Scheme 57.

Due to the initially disappointing results with the Boc anhydride mixed anhydride procedure, we examined an alternative approach that has also been reported to be effective for preparing the *tert* butyl esters of amino acids.

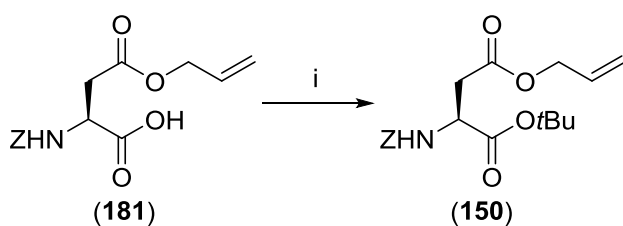
#### Using isopropenyl chloroformate

Zeggaf *et al.*,<sup>207</sup> have reported the esterification of the *N*-protected  $\alpha$ -amino acid using isopropenyl chlorocarbonate (IPCC) activation. The mixed anhydride generated in *situ* reacts with the alcohol in the presence of DMAP (Scheme 58).



**Scheme 58.** Mechanism of synthesis of  $\alpha$ -aspartate esters *via* IPCC/DMAP method.

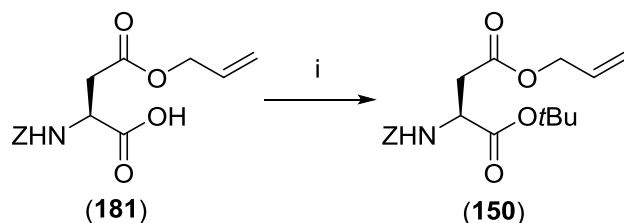
Two methods were attempted as described by Zeggaf *et al.*,<sup>207</sup> as outlined in Schemes 58 and 59.



Method C. *Reagents and conditions:* (i) IPCC, *t*BuOH, Et<sub>3</sub>N, DMAP, CH<sub>2</sub>Cl<sub>2</sub>, 35 °C, 16 h.

**Scheme 59.**  $\alpha$ -Esterification of Z-Asp(OAll)-OH.<sup>207</sup>

Firstly, Z-Asp(OAll)-OH (**181**) was treated with 1.1 eq of IPCC, in the presence of 1 eq of triethylamine and 3 eq of alcohol in CH<sub>2</sub>Cl<sub>2</sub>, at 35 °C. However only poor conversion to the ester was observed, plus several other products were visible on TLC. The crude ester (**150**) was isolated in only 10% yield (Scheme 59).



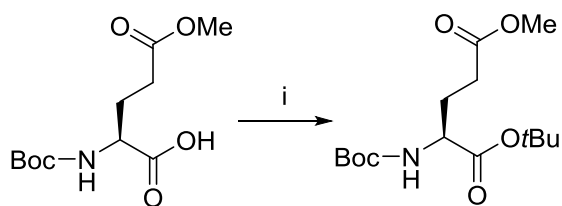
Method D. *Reagents and conditions:* (i) IPCC, *t*BuOH, Et<sub>3</sub>N, DMAP, 35 °C, 16 h.

**Scheme 60.**  $\alpha$ -Esterification of Z-Asp(OAll)-OH.<sup>208</sup>

In the previous example, the reaction was attempted at 35 °C, rather than room temperature as described by Zeggaf.<sup>208</sup> They also described esterification of some amino acids where *tert*-butyl alcohol is used as a solvent at 35 °C which has been shown to enhance the yield. We decided to apply the latter conditions, using the alcohol as a solvent. Furthermore, as the mixed anhydride prepared from IPCC releases the enolate of acetone, so having an excess of alcohol would favour the reaction. Compound (**181**) was subjected again to the esterification reaction but this gave the desired ester (**150**) in only a slightly improved yield (25%) as shown in Scheme 60.

#### *Using carbodiimide reagents*

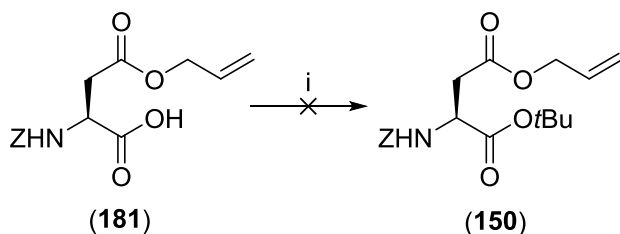
We used another method for mild one-pot esterification applicable to N-protected amino acids for the formation of the *tert* butyl ester (**150**). The acylation reaction again required one equivalent of DCC for one equivalent of carboxylic acid and a catalytic amount of DMAP, plus an excess of *tert*-butyl alcohol (Scheme 61).<sup>189</sup>



Reagents and conditions: (i) DCC, *t*BuOH, DMAP, rt 91%.<sup>189</sup>

**Scheme 61.**  $\alpha$ -Esterification using DCC-DMAP.

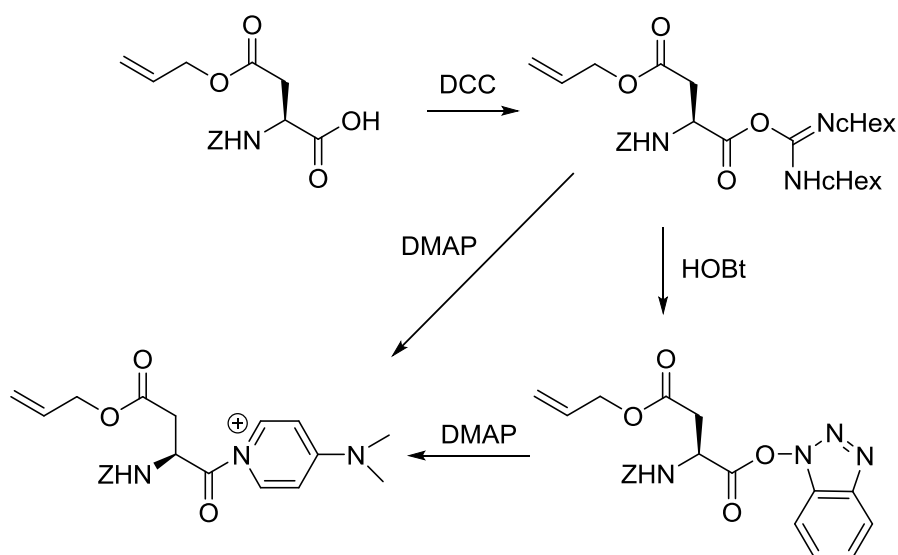
Compound (**181**) was first treated with 1.2 eq of DCC and then with 10 eq of *tert*-butyl alcohol in the presence of a catalytic amount of DMAP (Scheme 62). The reaction was unsuccessful and analysis by <sup>1</sup>H NMR on the crude material was not consistent with the expected product (**150**).



Method E. Reagents and conditions: (i) DCC, DMAP, *t*BuOH, 0 °C to rt, 16 h.

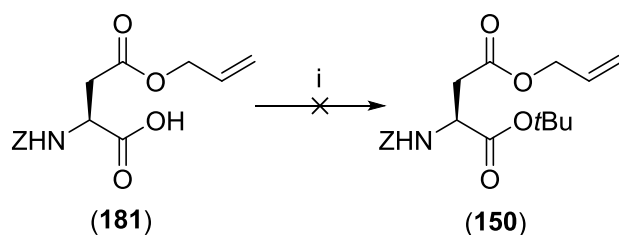
**Scheme 62.** Attempted  $\alpha$ -esterification of (**181**) using DCC-DMAP.

Based on the previous results with the use of DCC, we decided to use a method including HOBt, which should form a benzotriazole ester as intermediate, as shown in Scheme 63 .



**Scheme 63.** Mechanism of esterification with DCC-HOBt.





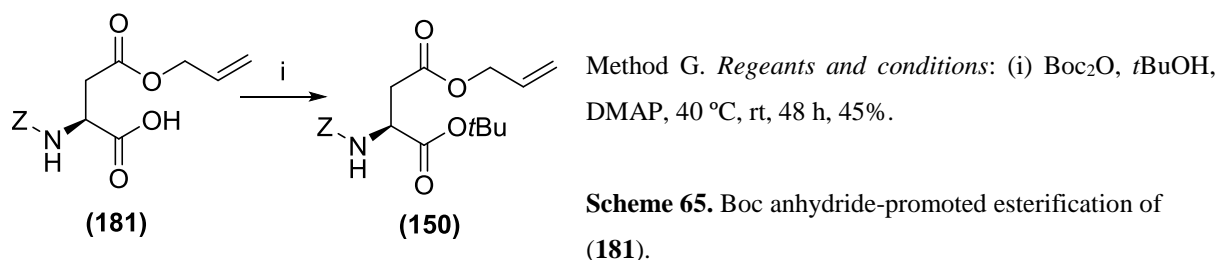
Method F. *Reagents and conditions:* (i) HOBt, DCC, DMAP, *t*BuOH, CH<sub>2</sub>Cl<sub>2</sub>/DMF, rt, 16 h.

**Scheme 64.** Attempted  $\alpha$ -esterification of **(181)** with DCC-HOBt.

Compound **(181)** was first treated with 1.4 eq of HOBt and 1 eq of DCC, then with 10 eq of alcohol in the presence of DMAP as the catalyst. However no ester **(150)** was formed and the starting material was recovered, as shown in Scheme 64.

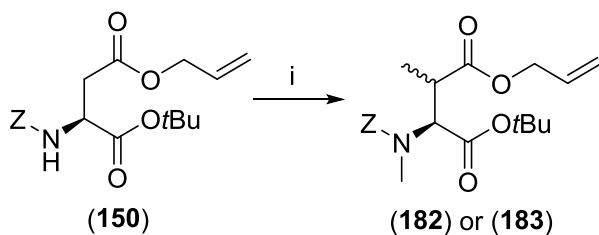
As the attempts with DCC were unsuccessful and the ipcc-promoted esterification of amino acids proved no more effective than the Boc<sub>2</sub>O method, we put all our effort into synthesizing compound **(150)** by the latter route.

After several attempts, the best conditions found to carry out the reaction with Boc anhydride were consistent with *tert*-butyl alcohol as a solvent in the presence of DMAP at 40 °C, for 48 h. This gave **(150)** in 45% yield, following column chromatography, plus unchanged starting material, as outlined in Scheme 65.



The next step was the alkylation of the  $\beta$ -enolate generated from the protected aspartic acid derivative. This was effected by reaction of **(150)** with iodomethane under basic conditions.

## Method A



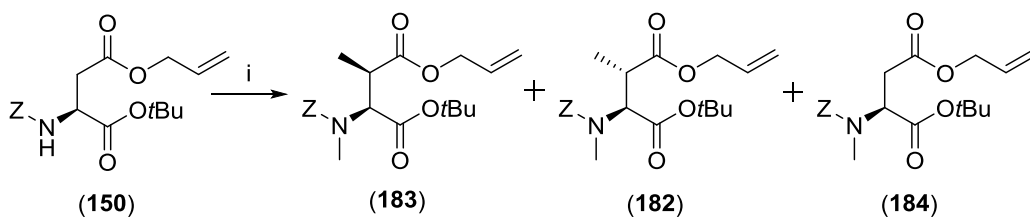
Method A. *Reagents and conditions:* (i) LiHMDS, THF, -78 °C, then MeI, warm up to rt, 18 h.

**Scheme 66.**  $\beta$ -Alkylation reaction on (150) according to Park *et al.*<sup>198</sup>

Compound (150) was subjected to the alkylation method used by Park *et al.*,<sup>198</sup> as it had been previously successful with (159a). The enolate dianion was obtained by treating (150) with 2 eq of lithium hexamethyldisilazide at -78 °C and was allowed to react with 1.2 eq of iodomethane. This gave a mixture of diastereoisomers of C+N-alkylated product (182) and (183), as shown in Scheme 66. These could not be separated by column chromatography in this initial study.

Because of these disappointing results, we then decided to apply directly the alkylation conditions reported by Yu.<sup>193</sup>

## Method B



Method B. *Reagents and conditions:* (i) LiHMDS, THF, HMPA, -78 °C 45 min, then MeI, warm up to rt, 16 h.

**Scheme 67.**  $\beta$ -Alkylation reaction on (150) according to Yu *et al.*<sup>193</sup>

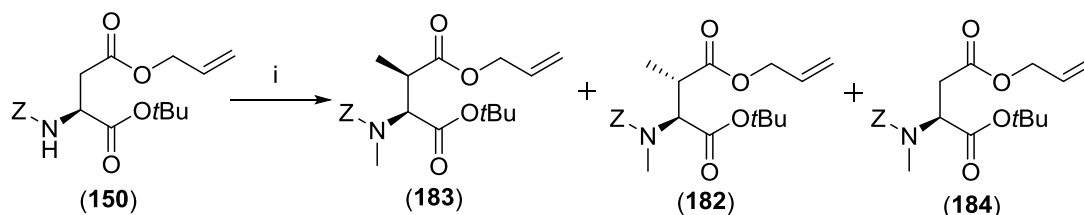
Following the method developed by Yu *et al.*,<sup>193</sup> the enolate dianion was obtained by treating (150) with 2 eq of lithium hexamethyldisilazide at -78 °C and 6.2 eq of HMPA. This was allowed to react with 3 eq of iodomethane. The methylation reaction gave 40% of the C+N-

alkylated product (**183**) isolated as a single diastereoisomer and a mixture of diastereoisomers of C+N-alkylated product (12%) (**182**), plus *N*-alkylated product (17%) (**184**), as shown in Scheme 67.

The stereochemical configuration of (**183**) was inferred from that of (**171**) for which the configuration has been determined unambiguously by Yu *et al.*<sup>193</sup> As in (**171**) and (**159a**) (both anti alkylation products), the H $\beta$  proton resonates at  $\delta$  *ca* 3.20. For the syn alkylation product (**159b**), the corresponding signal appears at  $\delta$  *ca* 3.00.

Beatty *et al.*,<sup>209</sup> have shown that the successful alkylation of Asp derivatives may be significantly affected by the temperature. We therefore speculated that the formation of the unwanted isomer, the *N*-alkylated product, might be avoided by stopping the alkylation at -78 °C, rather than allowing the reaction mixture to gradually warm to room temperature.

### Method C



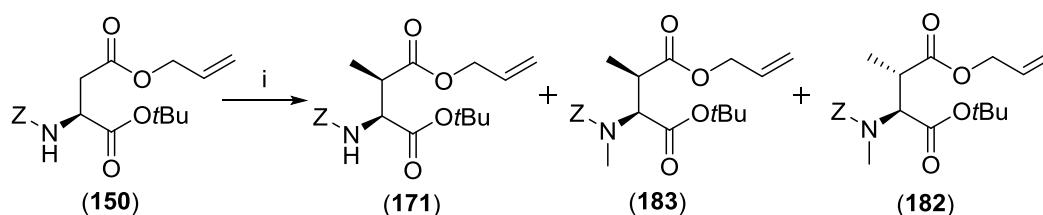
Method C. *Reagents and conditions:* (i) LiHMDS, THF, HMPA, -78 °C, 30 min, MeI, 3 h, then quench.

**Scheme 68.**  $\beta$ -Alkylation reaction on (**150**) – modification of Yu’s method.<sup>193</sup>

Compound (**150**) was alkylated under Yu’s procedure,<sup>193</sup> as outlined Scheme 68, with the modified quench procedure. However even with this precaution, we failed to reproduce the highly diastereoselectivity in the alkylation from Yu. The methylation reaction gave once again C+N-alkylated product (**183**), the diastereoisomeric C+N-alkylated product (**182**), plus *N*-alkylated product (**184**), with none of the desired compound observed, as shown in Scheme 68.

Prior to the next attempted alkylation, we ensured that all the reagents used were completely anhydrous, including the HMPA, which was dried over heated molecular sieves. Iodomethane was also distilled, although this precaution had not been necessary for the preparation of (**171a**).

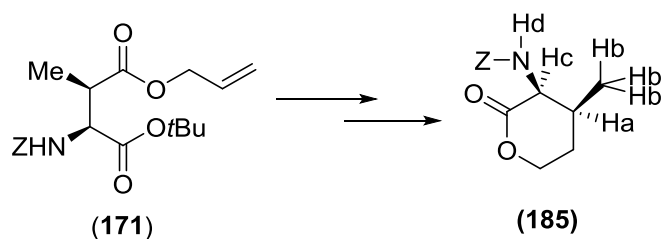
## Method D



Method D. *Reagents and conditions:* (i) LiHMDS, HMPA, THF, -78 °C, 30 min, MeI, 3 h, then quench.

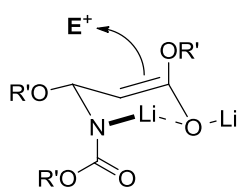
**Scheme 69.**  $\beta$ -Alkylation reaction of **(150)** – repeat of Method C with purified reagents.

The methylation reaction using distilled iodomethane and dried HMPA gave the desired compound **(171)** in 55% yield but still contaminated with a small amount of C+N-alkylated product **(183)**, as well as a mixture of the two C+N-alkylated diastereoisomers **(182)** and **(183)**, as outlined in Scheme 69. The  $^1\text{H}$  NMR of **(171)** isolated from this preparation was identical to that reported by Yu<sup>193</sup> and the optical rotation was also consistent with that reported previously. In Yu's study, the configuration of **(171)** was confirmed by conversion to the lactone **(185)**. The configuration was confirmed by  $^1\text{H}$  NMR NOESY experiments. Strong NOEs were observed between  $\text{H}_a$ - $\text{H}_c$  and  $\text{H}_b$ - $\text{H}_d$ , in the lactone which is consistent with the proposed configuration in **(171)**.<sup>193</sup>



**Scheme 70.** Proof of configuration of **(171)**.<sup>193</sup>

Reactions either using HPMA or not were therefore found to produce C- and N-alkylated product. The report of Yu<sup>193</sup> postulated that the stereoselectivity of the alkylation is driven by the 1,2-asymmetric induction of the enolate dianion from the aspartate derivative.



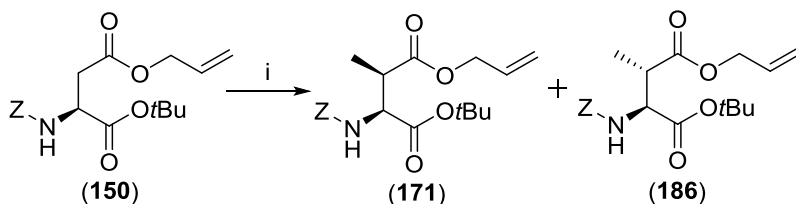
A chelated transition state which leads to the observed stereochemical outcome is possibly due to lithium- $\pi$ -olefin coordination based on a Zimmerman-Traxler-type model,<sup>210</sup> as shown in Figure 37.

**Figure 37.** Proposed chelated transition state in alkylation of (171).<sup>210</sup>

In contrast, Baldwin *et al.*,<sup>204</sup> have reported that the reaction of MeI with aspartate dianion gave a mixture of C- and N-alkylated products and the addition of HMPA to the reaction had no effect on the diastereomeric ratios. A similar study by Hanessian<sup>199</sup> also supports this observation.

After these disappointing results, it was decided to improve the method previously used for the synthesis of (171) without HMPA. The requirement here was to find the best alkylation procedure which would provide only the C-alkylated compound.

## Method E

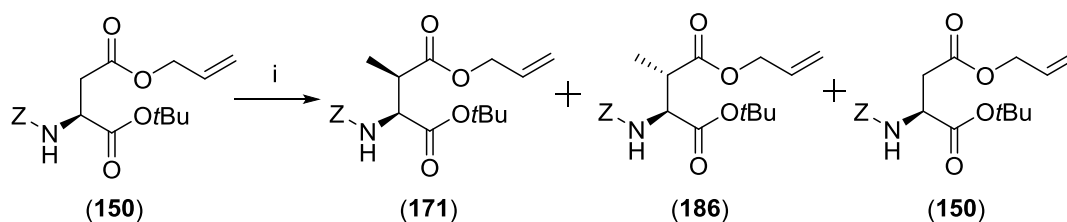


Method E. *Reagents and conditions:* (i) LiHMDS, THF, -78 °C, 45 min, MeI, 3 h, then quench.

**Scheme 71.**  $\beta$ -Alkylation reaction of (150) according to Yu *et al.*,<sup>193</sup> without HMPA.

Several alkylation reactions were carried out on different scales, using rigorously dried and distilled reagents. When 0.44 mmol of the ester (150) was used, a mixture of diastereoisomers in a ratio of 3:1 was obtained, which was separated by column chromatography. The compounds eluting from the column in order of increasing polarity were: 41% of the desired isomer (171) and then 17% of a 1:1 mixture with (186). The enolate dianion was obtained by treating (150) with 2 eq of lithium hexamethyldisilazide at -78 °C and was allowed to react with 3 eq of anhydrous iodomethane. On a 1.65 mmole scale, the crude material had the following composition: major/minor/starting material (2:1:0.5). The compounds were isolated by column chromatography eluting as follows: the C-alkylated product (171) in 48% yield, plus 200 mg

of mixture of (**171**) and diastereoisomer (**186**) (2:1), along with starting material (**150**), as shown in Scheme 72.



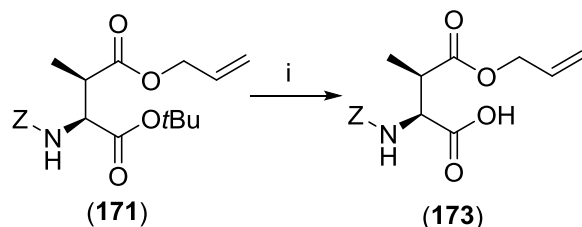
*Reagents and conditions:* (i) LiHMDS, THF, -78 °C, 45 min, MeI, 3 h, then quench.

**Scheme 72.** Large scale  $\beta$ -alkylation reaction of (**171**) under optimised conditions.

The preparation of (**171**) was thus achieved in moderate yield compared to the 91% yield reported by Yu.<sup>193</sup> Our results seem to rather correlate with the  $\beta$ -methylation of (**158**) to give compounds (**159a**) and (**159b**), reported by Park *et al.*<sup>198</sup>

Having successfully introduced the second stereocentre, (**171**) was ready to be used as a scaffold to build up the Amoa side chain. The next stage was to deprotect the  $\alpha$ -carboxyl group for further elongation of the molecule *via* the  $\alpha$ -aldehyde.

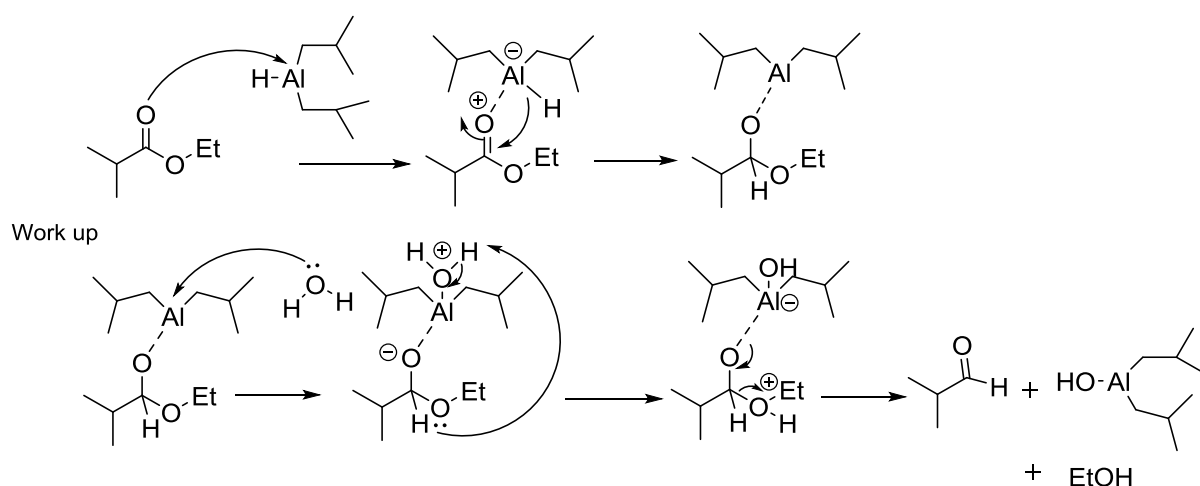
Unexpectedly the standard removal of the *tert*-butyl ester of (**171**) with 4M HCl in dioxane failed, giving the unchanged starting material, however the deprotection was effective using TFA/CH<sub>2</sub>Cl<sub>2</sub> (3:8, v/v) at room temperature, which gave the expected product (**173**) in 91% yield, as shown in Scheme 73.



*Reagents and conditions:* (i) TFA/CH<sub>2</sub>Cl<sub>2</sub> (v/v: 3:8), rt, 2 h.

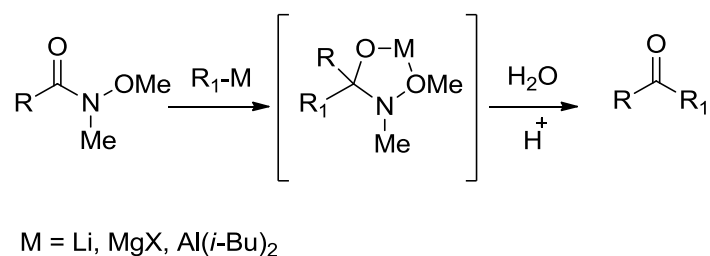
**Scheme 73.** Removal of *tert*-butyl ester of (**171**).

Esters and amides undergo reduction to the corresponding aldehydes using DIBAL-H at low temperature followed by acidic quench. The reactions occurs in three steps, first the lone pair of oxygen coordinates with aluminium, then the hydride is delivered to the carbonyl carbon, to give the tetrahedral intermediate, which is stable at low temperature and whose breakdown is finally achieved by quenching with water, as shown in Scheme 74.



**Scheme 74.** Reduction of an ester by DIBAL-H.

The development of *N*-methoxy-*N*-methyamides, Weinreb amides,<sup>211</sup> allows the conversion of carbonyl compounds at the carboxylic acid oxidation level to the corresponding aldehydes and ketones.<sup>212</sup> The efficiency of this transformation is attributed to the exceptional stability of the tetrahedral intermediate that is generated *in situ*, as outlined in Scheme 75.<sup>211</sup>

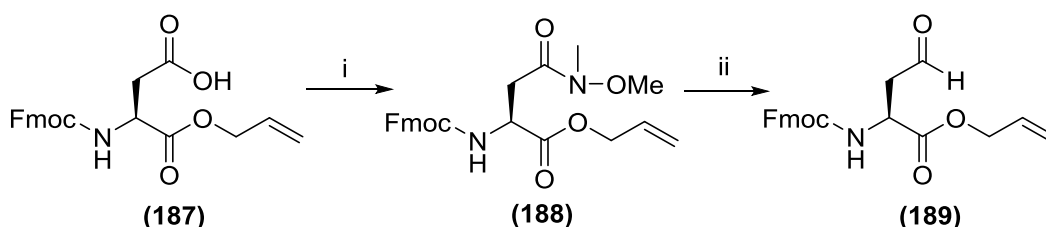


**Scheme 75.** Weinreb amide reduction by DIBAL-H.

Even with a large excess of the organometallic reagent, no over-addition occurs due to the chelation of a metal ion between the resulting alkoxide and the oxygen atom of the *N*-methoxy group. This prevents the premature release of the ketone functionality or formation of an iminium intermediate until the acidic work-up has been performed. This remarkable feature of

Weinreb amides allows them to be converted into aldehydes when treated with aluminium or boron hydrides, as shown in Scheme 75.<sup>213</sup>

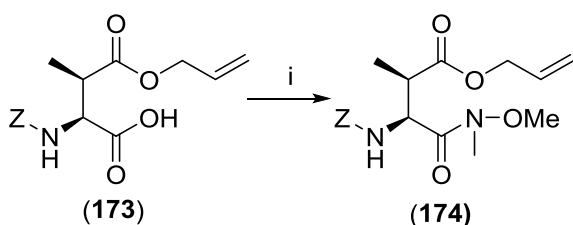
Bayer *et al.*,<sup>194</sup> have successfully achieved the preparation of an aspartic acid aldehyde (**189**) from the Weinreb amide (**188**) which was selectively reduced with DIBAL-H, as shown in Scheme 76.



*Reagents and conditions:* (i) HCl, HNOMeMe, NEM, PPA, CH<sub>2</sub>Cl<sub>2</sub>, rt; (ii) DIBAL-H, THF, -78 °C; then acidic work-up with KHSO<sub>4</sub>, 63% yield (over two steps).

**Scheme 76.** Preparation of the aldehyde using Weinreb amide.<sup>194</sup>

To apply this approach for our system, activation of the Asp derivative (**173**) with HOBt/EDC for conversion to the methoxymethylamide (**174**) was carried following the method of Spaltenstein, but using DIPEA in place of *N*-methylmorpholine (Scheme 77).<sup>214</sup>

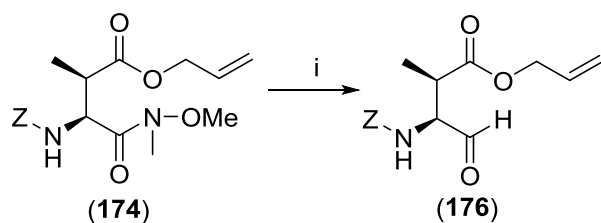


*Reagents and conditions:* (i) EDC.HCl, HOBt/H<sub>2</sub>O, CH<sub>2</sub>Cl<sub>2</sub>/DMF, then MeNHOMe.HCl, rt, 53% yield.

**Scheme 77.** Preparation of the Weinreb amide (**174**).<sup>194</sup>

The reduction of (**174**) was carried out with 1.2 eq of DIBAL-H and the reaction was monitored by TLC. After 2 h reaction, TLC showed starting material, therefore another 1.2 eq of DIBAL-H was added to the reaction mixture. After 2 h reaction, TLC showed partial consumption of the starting material, therefore another 0.44 eq of DIBAL-H was added to the reaction mixture. After 9 h reaction, TLC showed no further change (Scheme 78).



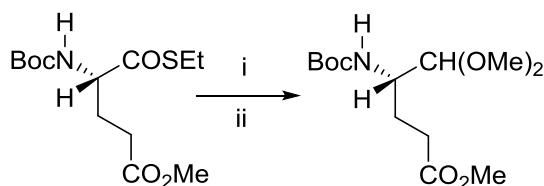


*Reagents and conditions:* (i) DIBAL-H, THF, -78 °C and then acidic quench.

**Scheme 78.** Attempted reduction of the Weinreb amide (174) with DIBAL-H.<sup>194</sup>

After work-up under acidic conditions, the crude  $^1\text{H}$  NMR spectrum revealed a mixture of the desired aldehyde (176) plus the unchanged starting material. The ratio of (176):(174) was approximately 1:3, as judged by  $^1\text{H}$  NMR. Unfortunately the aldehyde (176) co-eluted with the starting material (174), so it was not possible to separate them, after repeated attempts at column chromatography.

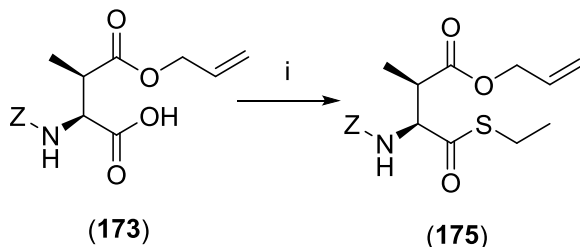
After this unsuccessful attempt to prepare the aldehyde (176), we then turned our attention to the reduction of a thiol ester. Fukuyama *et al.*,<sup>215</sup> have reported the reduction of ethyl thiol esters with triethylsilane and palladium on carbon. This method is suitable for the conversion of optically active amino acids into  $\alpha$ -amino aldehydes. As these intermediates are known to racemize even under mild conditions, they must be carefully handled or converted to more stable acetal derivatives (Scheme 79).



*Reagents and conditions:* (i)  $\text{Et}_3\text{SiH}$ , 10% Pd/C, acetone, rt; (ii) CSA, MeOH, 95%.

**Scheme 79.** Reduction of a thiol ester to the  $\alpha$ -aminoaldehyde using  $\text{Et}_3\text{SiH}$  and Pd/C.<sup>215</sup>

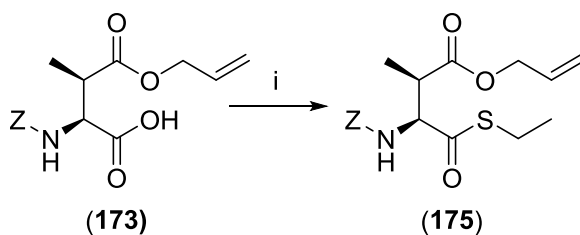
Compound (**173**) was first converted to the ethyl thiol ester (**175**) using DCC as an activating agent in the presence of a catalytic amount of DMAP. This gave the corresponding ester (**175**) in 52% yield, as shown in Scheme 80.



*Method A. Reagents and conditions:* (i) EtSH, DCC, DMAP, CH<sub>2</sub>Cl<sub>2</sub>, rt, 52%.

**Scheme 80.** Preparation of thiol ester (**175**) using DCC/DMAP activation.

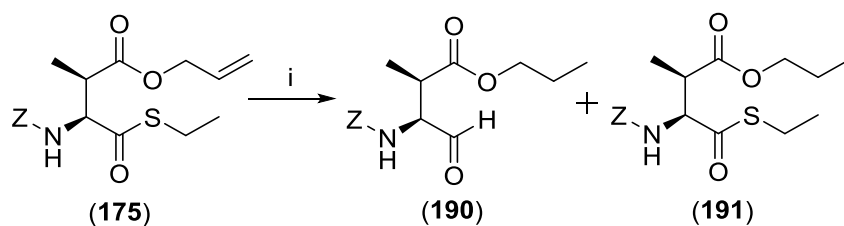
When the coupling was carried out with EDC.HCl and DMAP, the thiol ester (**175**) was obtained in 40% yield, a slightly lower yield compared to coupling with DCC.



*Method B. Reagents and conditions:* (i) EtSH, EDC.HCl, DMAP, DMF, 40%.

**Scheme 81.** Preparation of the thiol ester (**175**) using EDC.HCl, DMAP.<sup>215</sup>

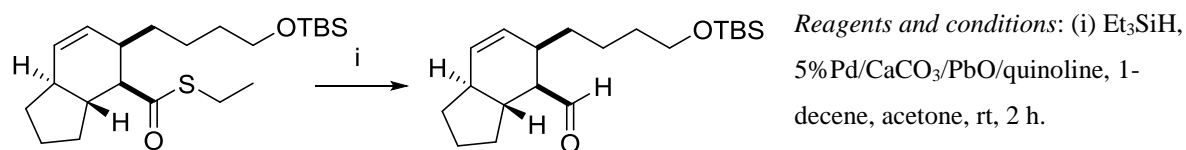
Compound (**175**) was subjected to reduction under Fukuyama conditions,<sup>215</sup> using 5 eq of Et<sub>3</sub>SiH and Pd/BaSO<sub>4</sub> (Scheme 82). After 2 h reaction all the starting material was consumed and the oil obtained was purified by column chromatography. This did not give the expected compound (**176**) but a mixture of two reduced compounds (**190**) and (**191**), which were separable by column chromatography. In order to prevent the reduction of the allyl group, a less reactive catalyst, Pd/BaSO<sub>4</sub>, was used in place of Pd/C employed by Fukuyama.<sup>215</sup> However, although the thiol ester group was reduced to the aldehyde function, olefin reduction could not be avoided. On prolonged reaction time (48 h), only the reduced aldehyde (**191**) was isolated but in very poor yield (7%).



*Reagents and conditions:* Method A: (i) Pd/BaSO<sub>4</sub>, Et<sub>3</sub>SiH, acetone, rt, 2 h; or Method B: as A, reaction for 48 h.

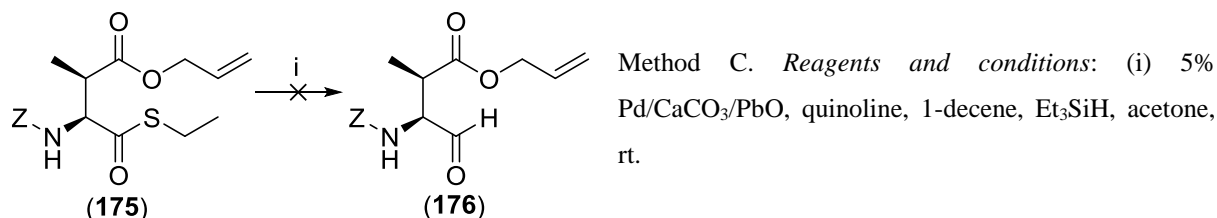
**Scheme 82.** Attempted reductions of the thiol ester (175).<sup>215</sup>

As Pd/BaSO<sub>4</sub> appeared to result in faster reduction of the allyl group of (175) than the thiol ester, in the next attempt, we tried Lindlar catalyst, Pd/CaCO<sub>3</sub>/PbO and a monosubstituted alkene as an additive in the reaction mixture. This was based on the work of Evans *et al.*,<sup>195</sup> who reported that the reduction of a thiol ester to the aldehyde using Pd(0)/triethylsilane led to concomitant reduction of an alkene within the same molecule. Subsequently they used an excess of sacrificial alkene, as shown in Scheme 83, to suppress this side reaction.



**Scheme 83.** Selective reduction of a thiol ester in the presence of an alkene according to Evans.<sup>195</sup>

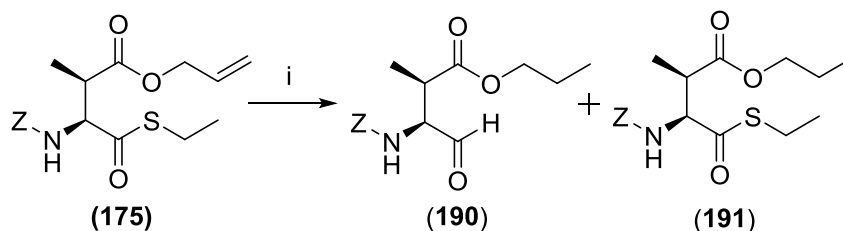
Following the method used by Evans *et al.*,<sup>195</sup> (175) was again subjected to selective reduction, in the presence of quinoline and 1-decene. This gave unchanged starting material, even when an excess of triethylsilane was used.



**Scheme 84.** Attempted reduction of (175) according to Evans' procedure.<sup>195</sup>

Attempts to reduce selectively (175) using a Pd catalyst/triethylsilane in the presence of quinoline and 1-decene were, therefore, unsuccessful. However, when we used only Pd/BaSO<sub>4</sub>

in the presence of 1-decene, the TLC revealed a mixture of the two reduced compounds (**190**) and (**191**) as shown in Scheme 85. No further investigation was carried out on this.



Method D. *Reagents and conditions:* (i) Pd/BaSO<sub>4</sub>, Et<sub>3</sub>SiH, 1-decene, acetone, rt, 2h.

**Scheme 85.** Further attempted selective reduction of (**175**).

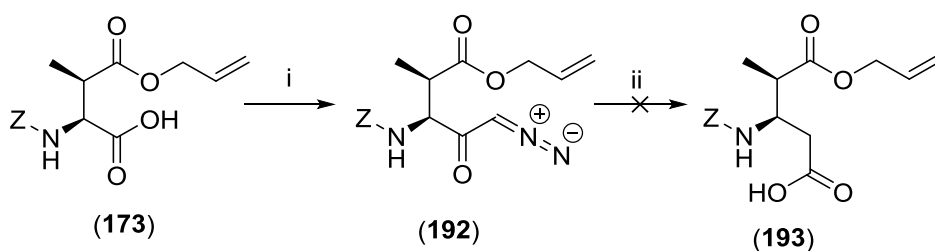
Because of the problems encountered in producing the required aldehyde (**176**) through the Weinreb amide (**174**) or selective reduction of thiol ester (**175**), this discouraged us from pursuing the synthetic approach using the  $\alpha$ -aldehyde (**176**). Subsequently we turned our efforts to producing the homologated carboxylic acid at (**173**) *via* a more conventional but slightly less direct route.

The Arndt-Eistert<sup>216</sup> procedure allows the conversion of activated carboxylic acids to diazoketones by the action of diazomethane, followed by Wolff rearrangement in the presence of nucleophiles such as water, alcohols or amines. The method is used for the synthesis of homologated carboxylic acids and their derivatives such as esters or amides. Recently this has become widely used in the synthesis of  $\beta$ -peptide and  $\beta$ -amino acid derivatives from the protected  $\alpha$ -amino acids.<sup>217</sup>

The transformation requires the use of diazomethane and since it is highly toxic, labile and explosive, a less hazardous substitute, trimethylsilyldiazomethane (TMSCHN<sub>2</sub>) has been developed by Aoyama and Shioiri.<sup>218</sup> The application of TMSCHN<sub>2</sub> in the classical Arndt-Einstert reaction starting from acyl chlorides is efficient. However, in the case of amino acid chemistry, other activation methods for activation, such as mixed anhydrides and the use of coupling reagents, are preferred.

Van Nguyen *et al.*,<sup>219</sup> have reported the Arndt-Eistert reaction using the activated ester formed by 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethylaminium tetrafluoroborate (TBTU), a coupling agent, followed by treatment with TMSCHN<sub>2</sub>.

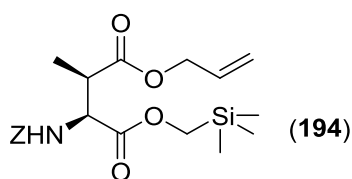
Following the method used by van Nguyen *et al.*,<sup>219</sup> (**173**) was activated with 1 eq of TBTU, followed by treatment with 2 eq of TMSCHN<sub>2</sub>. After 3 h 30 min reaction, TLC showed disappearance of the starting material and the resulting crude product was purified by column chromatography. This intermediate was subjected to Wolff rearrangement but the desired product (**193**) was not obtained, as outlined in Scheme 86.



*Reagents and conditions:* Method A: (i) TBTU, THF/DMF, DIPEA, 0 °C, 30 min, then TMSCHN<sub>2</sub>, 0 °C, 3 h 30 min; (ii) silver benzoate, dioxane:water (2:1), reflux 16 h.

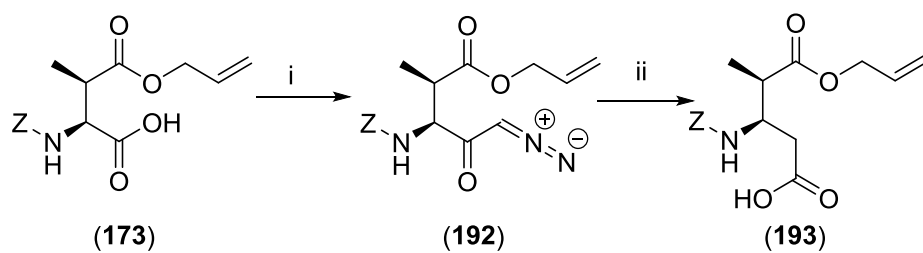
**Scheme 86.** Attempted Arndt-Eistert homologation of (**173**) using TBTU as coupling reagent.

Further examination of the <sup>1</sup>H NMR spectrum of the supposed diazomethylketone (**192**) showed an absence of the expected H<sub>β</sub> methane signal. We speculated that the intermediate formed might in fact be the trimethylsilylmethyl ester derivative (**194**) isolated after silica gel chromatography eluting with methanol, as shown in Figure 38.<sup>220</sup>



**Figure 38.** Trimethylsilylmethyl ester derivative formed during attempted Arndt-Eistert synthesis.<sup>220</sup>

Compound (**173**) was again subjected to an Arndt-Eistert synthesis but this time using the method of Patil *et al.*<sup>217</sup> Compound (**173**) was activated as the mixed anhydride, prepared with ClCOOEt/DIPEA and was allowed to react with 2 eq of TMSCHN<sub>2</sub>. This was followed by the Ag<sup>+</sup>/base-catalysed Wolff rearrangement of diazoketone (**192**) in water, which proceeded smoothly to give the homologated β-amino acid (**193**) in 27% yield.

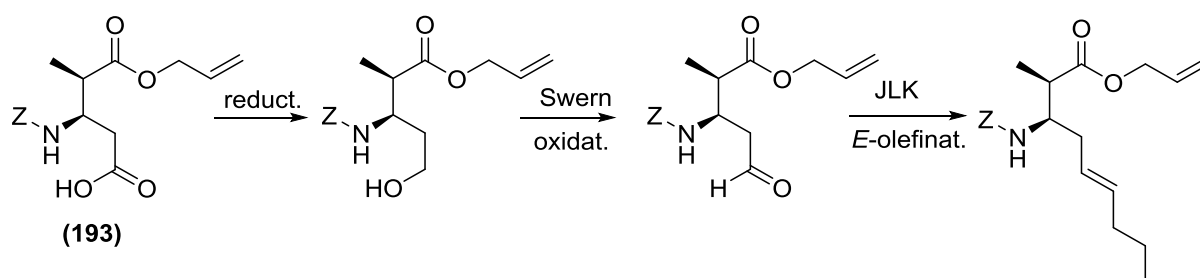


*Reagents and conditions:* Method B: (i) ClCOOEt, DIPEA, THF, -15 °C to -4 °C, 30 min, then TMSCHN<sub>2</sub>, 4 °C, 48 h; (ii) silver benzoate, dioxane:water (2:1), reflux, 16 h.

**Scheme 87.** Arndt-Eistert synthesis with (173) using ClCOOEt/DIPEA as a coupling reagent.<sup>217</sup>

### 5.3.7. Conclusion and future work

A selectively protected  $\beta$ -methyl-substituted Asp derivative (**173**) was successfully prepared, suitable for the incorporation of the Amoa side chain. The key step of homologating the  $\alpha$ -carboxylic function has been achieved by Arndt-Eistert chemistry. The next steps would be to convert the new carboxylic function to the corresponding aldehyde. This might be achieved *via* the anhydride, followed by reduction to the primary alcohol and then selective re-oxidation. The alkene function might then be introduced by *E*-selective Julia-Kocienski olefination to give the protected Amoa diastereoisomer. This chemistry is outlined in Scheme 88.



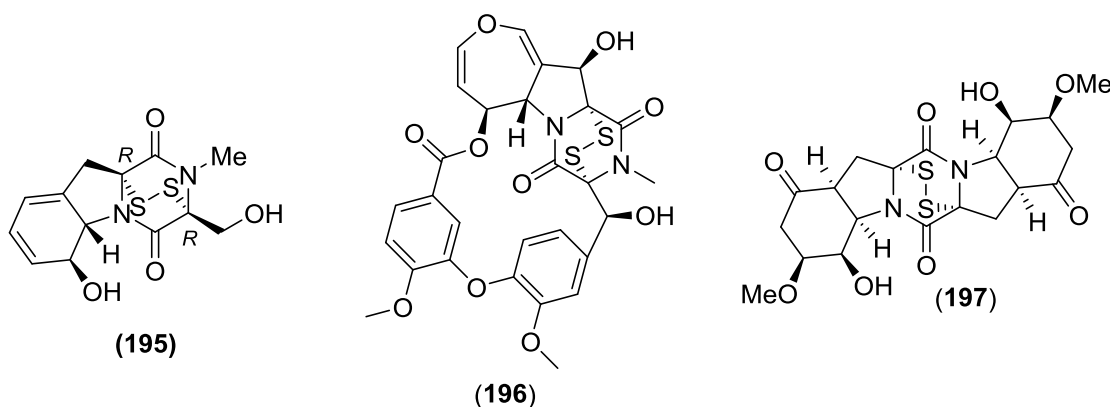
**Scheme 88.** Planned synthetic route to protected Amoa diastereoisomer.

Using this overall approach, all four Amoa diastereoisomers may now be prepared, starting from L- or D-aspartic acid as appropriate. Selective deprotections and Fmoc protection of the *N*- $\alpha$  function would then finally provide derivatives suitable for Fmoc SPPS.

## 5.4. 2,5-Diketopiperazines : proline-containing cyclodipeptides as potential Family 18 chitinase inhibitors.

### 5.4.1. DKP in nature

Innumerable natural product molecules containing the 2,5-diketopiperazine (2,5-DKP) ring have a wide range of interesting biological activities, such as antitumour,<sup>221</sup> antimicrobial<sup>222</sup> and antiviral<sup>223</sup> activities. The sulfur-bridged 2,5-DKPs are a class of metabolites with epipolythiodioxopiperazine (ETP) structures and are mainly found in fungi and lichens, as shown in Figure 39.<sup>224</sup> The toxicity of these ETPs rings in 2,5-DKP is due to the presence of the disulfide bridge which can inactivate proteins *via* reaction with a thiol group and by generation of reactive oxygen species by redox cycling.<sup>225</sup>



**Figure 39.** Natural 2,5-DKP- and epithiodiketopiperazines

The best known ETP is the lipid-soluble 2,5-DKP gliotoxin (**195**) which has toxic effects on phagocytic cells and T-lymphocytes at low concentrations *in vitro*.<sup>226</sup> The 15-membered antifungal macrolide MPC1001 (**196**) possesses antiproliferative activity against the human prostate cancer line (DU14S) with an  $IC_{50} = 9.3$  nM.<sup>227</sup> Rostratin (**197**) showed *in vitro* cytotoxicity against human colon carcinoma (HCT-116) with an  $IC_{50} = 1.57$   $\mu$ M.<sup>228</sup>

### 5.4.2. Diketopiperazines as potential drug leads

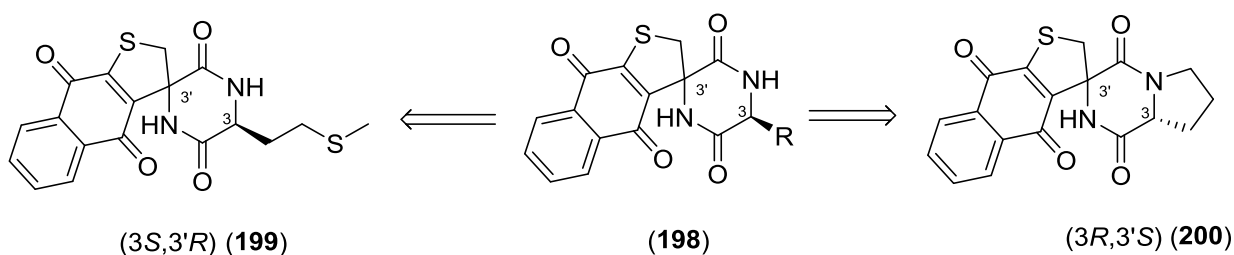
The 2,5-DKPs are a class of compounds that are useful in medicinal chemistry, combinatorial chemistry and targeted organic chemistry. This is because these small cyclic dipeptides are conformationally rigid chiral templates with multiple H-bond acceptors and donor functionality. Furthermore they possess multiples sites for structural elaboration of diverse



functional groups with defined stereochemistry. In this regard, SAR has been explored in many 2,5-DKP templates to move from a lead to a clinical drug.

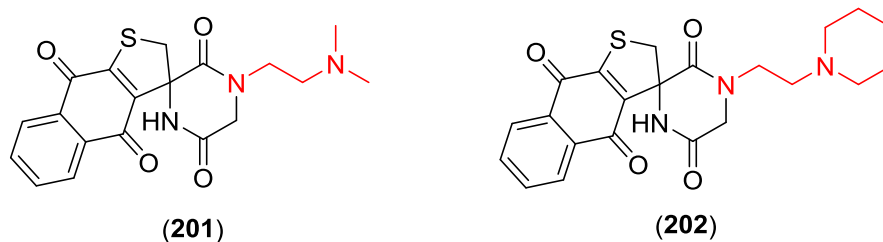
Cyclic dipeptides, DKPs, have been developed to obtain DNA-binding agents that overcome multidrug resistance, as shown by the quinone-based anticancer agents. Tryptophan is commonly incorporated into 2,5-DKP natural products based on the tryptophan-proline, cyclo (L-Trp-L-Pro) moiety found in the tryprostatin mammalian cell-cycle inhibitors and the fumitremorgin multidrug-resistant transport inhibitors, which are possible candidates for the development of anticancer drugs.<sup>229, 230</sup> In addition, 2,5-DKP cores are found in naturally occurring peptide antibiotics.

*DKP Derivatives as DNA-Binding agents:*



**Figure 40.** Spirodiketopiperazine (198) and its derivatives.

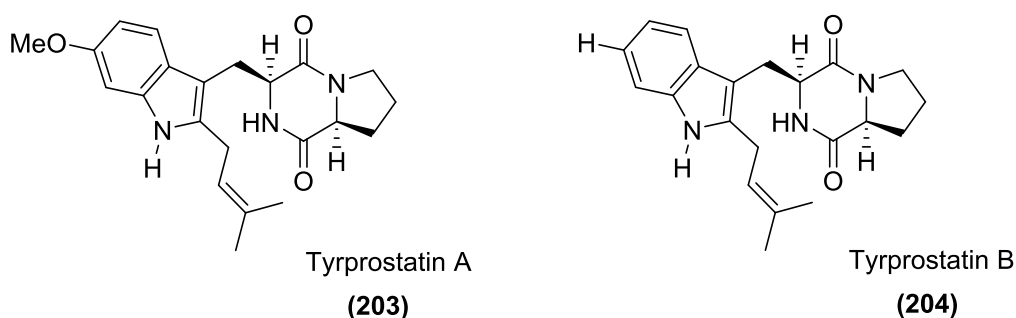
DNA-binding agents that exhibit selective cytotoxicity are potential leads for the treatment of cancer. The spirodiketopiperazines (198) have been developed as potentially new anticancer agents related to the quinone-based anticancer agents, doxorubicin and mitoxantrone and they are active against multidrug resistant tumour cell lines, unlike the parent compound.<sup>231</sup> SAR studies on these compounds showed that substitution at the C3-position and the stereochemical configuration at C3 and C3' were important in order to obtain high potency. Preparation of derivatives of (198) substituted at the C3-position showed that the (3*S*,3'*R*) (199) isomer derived from L-Met and the (3*R*,3'*S*) (200) isomer from D-Pro had cytotoxicity comparable or greater than that of doxorubicin against human breast and colon carcinoma cell lines, with (200) being the most potent across both cell lines.<sup>231</sup> Furthermore substitution on the nitrogen showed that (201) and (202) were the most promising compounds, with activity comparable to that of doxorubicin (Figure 41).<sup>231</sup>



**Figure 41.** Spirodiketopiperazine derivatives.

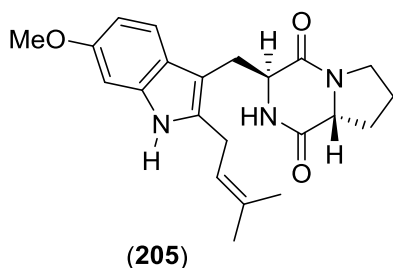
All these compounds (**199-202**) showed the ability to overcome potential multiple drug-resistance mechanisms, which was demonstrated by their high cytotoxicity against doxorubicin- and cisplatin-resistant cell lines. Their mode of binding to DNA was determined by STD-NMR spectroscopy which showed that the interaction with DNA occurs in a dual-binding mode: first the tricyclic planar core binds with DNA by intercalation and then the 2,5-diketopiperazine ring and its substituents bind externally to the double-stranded DNA.<sup>231</sup>

#### *DKP: Cell-cycle inhibitors*



**Figure 42.** Tryprostatins A and B.

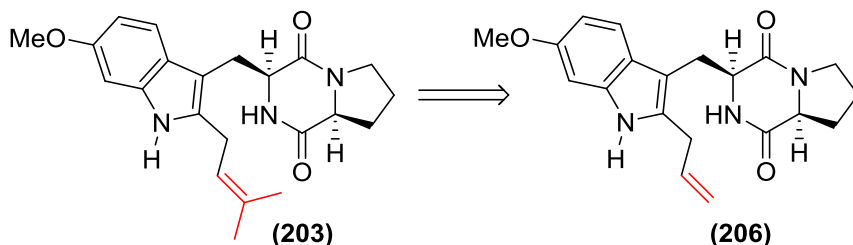
The tryprostatins A (**203**) and B (**204**) were isolated from a marine fungal strain of *Aspergillus fumigatus* and have attracted attention as potential anticancer drugs.<sup>229</sup> Compound (**204**) for example is a mammalian cell-cycle inhibitor,<sup>229</sup> whereas (**203**) is an inhibitor of the multidrug-resistance protein (BCRP/AGCG2) that mediates resistance to chemotherapeutics in breast cancer.<sup>230</sup> All four diastereoisomers of both tryprostatin A (**203**) and B (**204**) have been evaluated against human breast, prostate and lung cancer cell lines.



**Figure 43.** Tryprostatin C with D-Pro-L-Trp configuration.

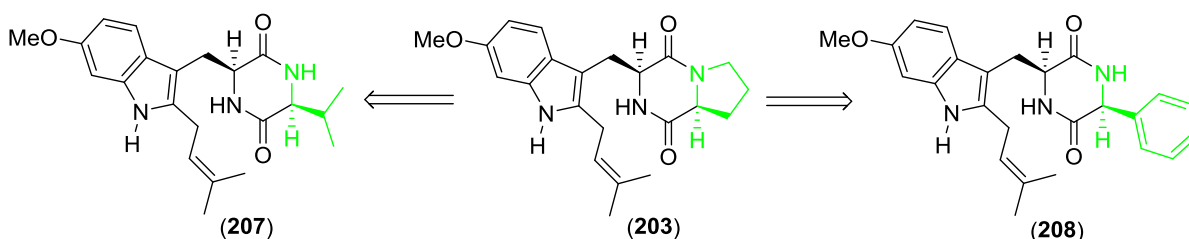
Only the DL diastereoisomer (205) of tryprostatin A showed potent cytotoxicity against all the cancer cell lines.<sup>229</sup> The L-Trp unit was clearly required because none of the other tryprostatins that contained the D-Trp unit showed activity.

Further investigation included the *N*-mono- and dialkylated indole ring derivatives of tryprostatin A (204). Replacing the isopropenyl substituent in (204) ( $IC_{50} = 68 \mu M$ ) gave (206) ( $IC_{50} = 60 \mu M$ ) with a similar level of potency towards inhibition of the growth of tsFT210. The benzyl analogue was inactive however.<sup>232</sup>



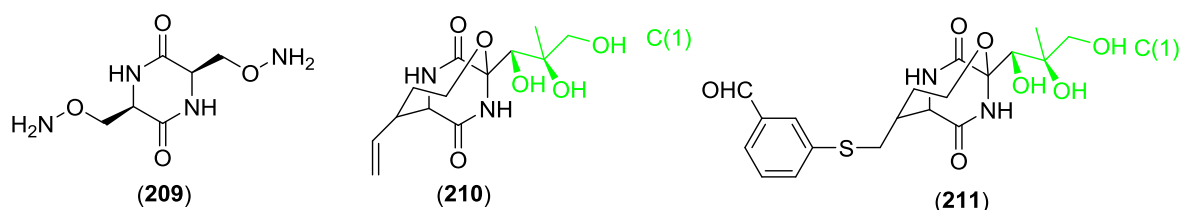
**Figure 44.** Tryprostatin A analogues: modification at the indole ring.

Substitution of the L-proline ring gave the most promising compounds in these series, with L-valine and L-phenylalanine, analogues (207) ( $IC_{50} = 19 \mu M$ ) (208) ( $IC_{50} = 10 \mu M$ ), being 3.5-fold and 7-fold more potent, respectively, than (203) ( $IC_{50} = 68 \mu M$ ).<sup>232</sup>



**Figure 45.** Tryprostatin A analogues: modification of the L-proline ring.

## DKP: Antibiotics and Antibacterials



**Figure 46.** DKP structure in natural peptide antibiotics.

DKP structures are found among several naturally occurring peptide antibiotics. They range from simple cyclic dipeptides to more complex fused structures. For instance, the simple antibiotic structure, the cycloserine dimer (**209**), showed activity against *Mycobacterium tuberculosis*.<sup>233</sup> The highly complex DKP derivative bicyclomycin (**210**) was isolated from *Streptomyces sapporonesis* and inhibits a broad spectrum of Gram-negative bacteria and the Gram-positive bacterium, *Micrococcus luteus*.<sup>234</sup> It possesses a low toxicity and it is rapidly absorbed in man when given intramuscularly. This antibiotic (**210**) (bicyclomycin) is used clinically to treat gastroenteritis. Bicyclomycin is the only known selective inhibitor of rho, a RecA-type ATPase. A crystal structure of bicyclomycin-rho complex was obtained in order to design new synthetic derivatives of bicyclomycin. SAR studies showed that the C(1) triol and [4.2.2]-bicyclic ring were essential for the bicyclomycin-rho inhibitory activity, whereas the C(5)-C(5a) exomethylene moiety was not necessary. Recently the bicyclomycin derivative, 5a-(3-formylphenylsulfanyl)dihydrobicyclomycin (**211**) was shown to inhibit rho by an order of magnitude more efficiently than (**210**).<sup>235</sup>

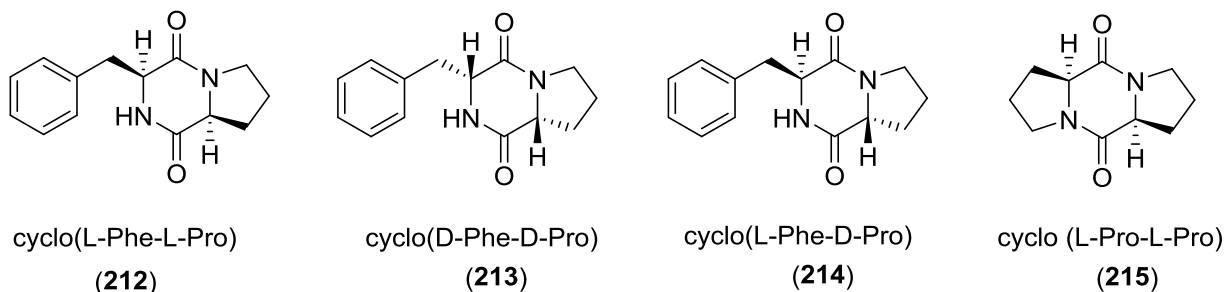
Many Gram-positive and Gram-negative bacteria communicate *via* the production and sensing of small, diffusible signal molecules that coordinate virulence-determinant production and regulate expression of specific genes responsible for communal behaviour. This is known as quorum sensing (QS).<sup>236</sup> Recently the 2,5-DKP based (cyclic Xaa-Pro) have been shown to both activate and inhibit (QS) in certain Gram-negative bacteria.<sup>222</sup>

### 5.4.3. Proline-containing DKP derivatives and their biological activities

Proline-based 2,5-DKPs are widely distributed in nature because the peptide bond to the proline amino acid residue has significant *cis* character favouring the intramolecular cyclization of Xaa-

Pro. Indeed many diketopiperazines with important biological properties contain a proline moiety.<sup>237</sup>

#### Antimicrobial

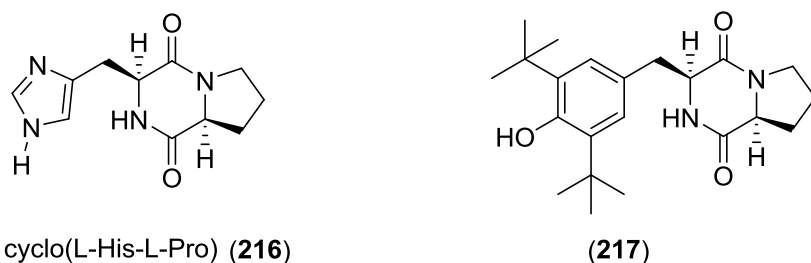


**Figure 47.** Proline-based DKPs with antimicrobial activity.

Cyclo(L-Phe-L-Pro) (**212**) is produced by the lactic acid bacteria *L. coryniformis* and *L. plantarum* and has been shown to possess antifungal activity against *A. fumigatus*. Its enantiomer (**213**), produced by a marine bacterium, inhibits the growth of the fish pathogen *Vibrio anguillarum* and was also shown to have antifouling activity. Furthermore (**212**) was active as a signal molecule, controlling the expression of genes important for the pathogenicity of the bacteria *Vibrio spp.*<sup>238</sup> The isomer cyclo(L-Phe-D-Pro) (**214**), isolated from *Bacillus subtilis sp.*, has been shown to be active against *V. anguillarum*.<sup>239</sup>

The symmetrical cyclo(L-Pro-L-Pro) DKP (**215**) was isolated from the bacterium *Pseudoalteromonas haloplanktis* TAC125 and the blowfly *Lucillia sericata* and has been shown to have weak antibacterial activity against *Pseudomonas auruginosa* and *M. luteus*.<sup>240</sup>

#### Neuroprotective agents



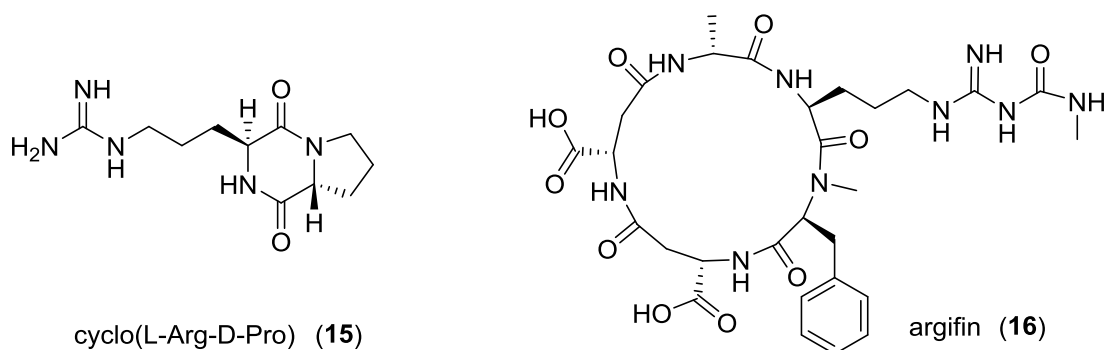
**Figure 48.** Proline-based DKPs examined as neuroprotective agents.

Cyclo(L-His-L-Pro) (**216**) is a metabolite from tryptropin-releasing hormone (TRP) in mammals and is a part of the complicated neuroendocrine control system. This facilitates central dopamine and suppresses food intake. It has been shown that (**216**) has neuroprotective effects in PCI2 cells stressed by serum deprivation and can prevent neuronal death induced by free radicals and calcium mobilization.<sup>241</sup> Due to the effect of imidazole acting as a scavenger of reactive oxygen species, a novel 2,5-DKP (**217**) based on cyclo(L-His-L-Pro) was designed. The imidazole moiety was replaced by a di-*tert*-butylphenol, as such phenols have the ability to act as scavengers for reactive oxygen species (ROS) in order to increase the neuroprotective effect of these DKPs.<sup>242</sup>

#### *Antifungal chitinase inhibitors*

Cyclo(L-Arg-D-Pro) CI-4 (**15**), was isolated from a marine bacterium *Pseudomonas sp.* and has biological activity against yeast cell separation and also against human pathogenic fungi. For instance, it has been found to inhibit chitinase activity and normal cell separation of *S. cerevisiae*. Its isomer cyclo(L-Arg-L-Pro) (**218**) had comparable modest enzyme activity against chitinase from *Bacillus sp.* whereas its isomer cyclo(D-Arg-L-Pro) (**219**) was much less active. A similar activity profile was obtained against *S. cerevisiae* with these three isomers. A crystal structure of the complex of (**15**) in SmChiB showed that (**15**) inhibits Family 18 chitinases, with its bicyclic structure mimicking the reaction intermediate of substrate-assisted catalysis.<sup>243</sup>

As it has already discussed in Section 1 CI-4, (**15**) is an interesting lead chitinase inhibitor, due to its low molecular weight, simple structure and drug-like properties compared to other known inhibitors. It inhibits SmChiB competitively with  $K_i = 0.65$  mM.<sup>141</sup>



**Figure 49.** Antifungal chitinase inhibitors.

CI-4 (**15**) and argifin (**16**) have comparable binding efficiency indices (BEI) of 2.58 and 6.31, respectively.<sup>244</sup> Although these cyclic dipeptides are weak inhibitors, they are potential starting scaffolds to elaborate new classes of chitinase inhibitors. Studies by Houston *et al.*,<sup>140</sup> have demonstrated that changing the stereochemical configuration of the proline ring changes the orientation of binding of the DKP but approximately similar interactions with the key active site residues are maintained. The arginine side in the CI-4 structure is less ordered and does not make any significant interaction within the chitinase, so this could be a site for derivatization and/or a change of configuration. By retaining the cyclic dipeptide substructure and extending the molecule along the groove-shaped active site, which is so effectively occupied by allosamidin, an increase in favourable interactions with the enzyme might then be achieved. Such extensions should be composed of groups that mimic the interaction between the *N*-acetylallosamine sugars of allosamidin and the -2/-3 subsites, establishing stacking interactions with the solvent-exposed aromatic residues. In support of this idea, crystal structure analysis on cyclo(L-Arg-L-Pro) (**218**), cyclo(L-His-L-Pro) (**216**), cyclo(L-Tyr-L-Pro) (**220**) and cyclo(Gly-L-Pro) (**221**) in *SmChiB*, showed that the common (**221**) substructure is sufficient for binding.<sup>140</sup> These studies suggested that modification of the side-chain of the non-proline residue could be a possible way to improve potency against chitinase activity in the design of new 2,5-DKPs based on a proline residue. In this series, (**216**) showed significant activity but only comparable to CI-4 itself with an IC<sub>50</sub> = 1.2 mM.

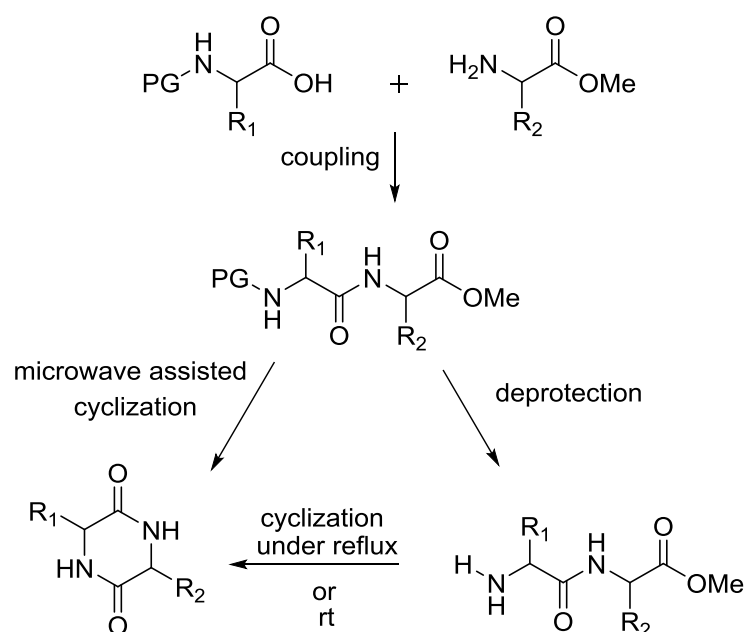
In original studies on CI-4 (**15**) by Izumida *et al.*<sup>139</sup> the D-D enantiomer of CI-4 was not explored. However studies in our laboratory<sup>245</sup> have shown that this compound and its protected analogue are surprisingly effective inhibitors. Based on this observation, some new cyclo (Xaa-Pro)-based dipeptides were designed, with different Xaa such as L/D-Pro, Gly, L-Ser, D-Arg, D-His, D-Phe. Protected Arg(Pmc), His(Trt) and Ser(Bn) were chosen in order to potentially increase active site interactions with the bacterial chitinases, by forming  $\pi$ - $\pi$  stacking interactions with conserved tryptophan residues within the chitinase activesite.

#### 5.4.4. Approaches to the synthesis of 2,5-diketopiperazines

##### *Dipeptide formation and cyclization*

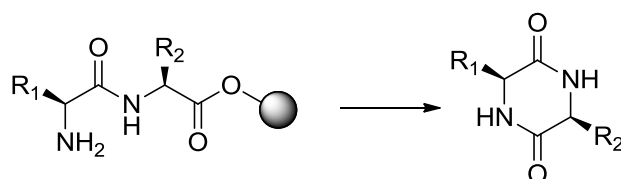
At its simplest, the preparation of a DKP involves the formation of a dipeptide between an *N*-protected  $\alpha$ -amino acid and  $\alpha$ -amino acid ester, then the deprotection of the dipeptide to give a

dipeptide ester with a free *N*-terminus. To achieve the cyclization, the amide bond must assume the *cis*-configuration. Most often the cyclization is carried out by heating in an inert medium, such as MeOH, toluene or DMF. Recently an efficient and environmentally benign one-pot solution-phase synthesis of 2,5-DKPs using microwave-assisted heating in water has been reported.<sup>246</sup> In this route the *N*-deprotection occurs *via* microwave irradiation followed by the cyclization, giving high yields of 2,5-DKP, as shown in Scheme 89.



**Scheme 89.** Dipeptide formation and microwave-assisted cyclization to DKPs.

#### *Cleavage-induced cyclization*



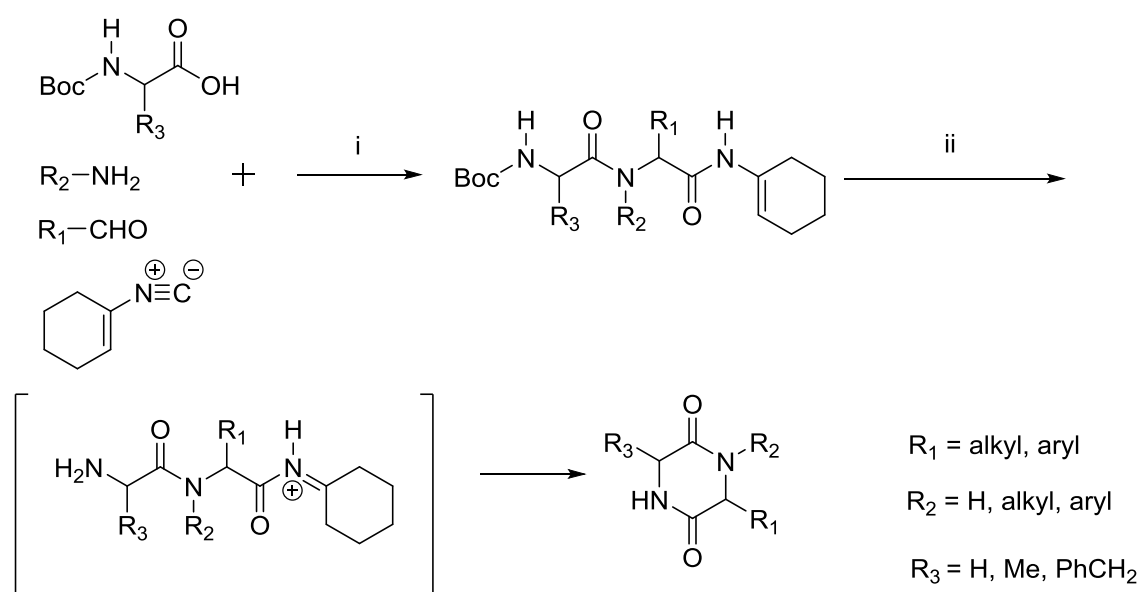
**Scheme 90.** DKP formation upon cleavage-induced cyclization.

The cleavage-induced cyclization of linear dipeptides is one the most common strategies for the solid-phase synthesis of 2,5-DKP libraries. High yields of cyclic dipeptides have been obtained using both thermal and microwave-assisted heating. The PEGA-Ser resin was found to give high yields of 2,5-DKPs in water with microwave-assisted heating for 30 min.<sup>247</sup>



### Ugi chemistry

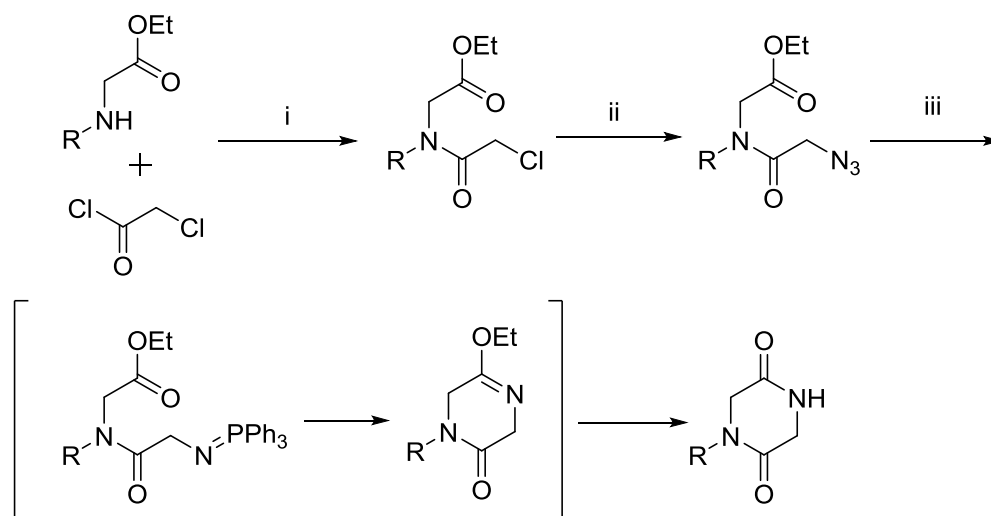
Preparation of DKP by the Ugi reaction uses an isonitrile, a Boc-protected amino acid aldehyde and an amine (Scheme 91). The main advantage of this approach is that it does not require the use of homochiral amino acids and coupling reagents, as in the peptide coupling routes above and the reactant amines, carboxylic acids and aldehydes are usually all readily available. The terminal amide produced from the isonitrile moiety is often removed during the formation of the 2,5-DKP ring.<sup>248</sup>



Reagents and conditions: (i) MeOH, rt; (ii) 10% TFA/DCE.

**Scheme 91.** Preparation of DKPs by Ugi reaction.

### Aza-Wittig cyclization

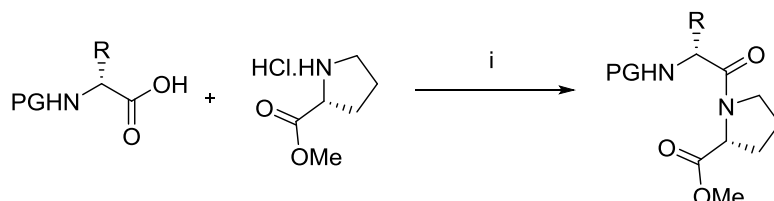


Reagents and conditions: (i)  $\text{K}_2\text{CO}_3$ ,  $\text{CH}_2\text{Cl}_2/\text{H}_2\text{O}$ ; (ii)  $\text{NaN}_3$ , DMF; (iii)  $\text{PPh}_3$ , THF, rt.

**Scheme 92.** Aza-Wittig cyclization route to DKPs.<sup>249</sup>

An intramolecular aza-Wittig reaction followed by hydrolysis of the resulting imine ether has been used to prepare the 2,5-DKP ring as shown in Scheme 92. Acylation of the amino acid with chloroacetyl chloride gives the azide, following displacement with sodium azide. This is then converted into an iminophosphorane intermediate. This undergoes cyclization at C-2 to give the ester imino ether which, in the presence of moist THF, gives a good yields of 2,5-DKP.<sup>249</sup>

For our work the CI-4 analogues were prepared *via* the dipeptide ester cyclization route. A linear dipeptide was first prepared by coupling proline methyl ester to the corresponding Fmoc amino acid or Boc amino acid using PyBOP or EDC/HOBt as activating agents (see Scheme 93).



Reagents and conditions: (i) coupling reactions in  $\text{CH}_2\text{Cl}_2$  or DMF/ $\text{CH}_2\text{Cl}_2$ .

**Scheme 93.** Synthesis of dipeptide intermediates for CI-4 derivatives.

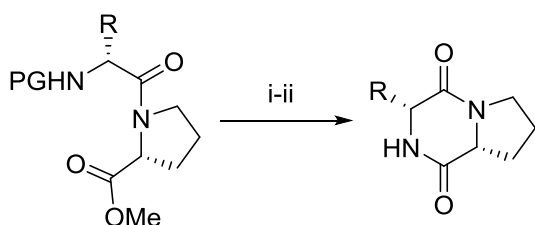
**Table 5.** Preparation of dipeptides

<b>PG-Xaa</b>	<b>Pro-OMe</b>	<b>PG-Xaa-Pro-OMe</b>	<b>Cond.</b>	<b>Yield%</b>
Fmoc-D-Pro-OH ( <b>222</b> )	D-Pro-OMe ( <b>223</b> )	Fmoc-D-Pro-D-Pro-OMe ( <b>224</b> )	a	59
Fmoc-L-Pro-OH ( <b>225</b> )	L-Pro-OMe ( <b>226</b> )	Fmoc-L-Pro-L-Pro-OMe ( <b>227</b> )	a	51
Fmoc-Gly-OH ( <b>228</b> )	D-Pro-OMe ( <b>223</b> )	Fmoc-Gly-D-Pro-OMe ( <b>229</b> )	b	57
Boc-L-Pro-OH ( <b>230</b> )	L-Pro-OMe ( <b>226</b> )	Boc-L-Pro-L-Pro-OMe ( <b>231</b> )	b	60
Boc-D-Pro-OH ( <b>232</b> )	D-Pro-OMe ( <b>223</b> )	Boc-D-Pro-D-Pro-OMe ( <b>233</b> )	b	51
Boc-Gly-OH ( <b>234</b> )	D-Pro-OMe ( <b>223</b> )	Boc-Gly-D-Pro-OMe ( <b>235</b> )	b	42
Boc-D-Ser(OBn)-OH ( <b>236</b> )	D-Pro-OMe ( <b>223</b> )	Boc-D-Ser(OBn)-D-Pro-OMe ( <b>237</b> )	b	75
Boc-L-Ser(OBn)-OH ( <b>238</b> )	L-Pro-OMe ( <b>226</b> )	Boc-L-Ser(OBn)-L-Pro-OMe ( <b>239</b> )	b	83
Fmoc-D-His(Trt)-OH ( <b>240</b> )	D-Pro-OMe ( <b>223</b> )	Fmoc-D-His(Trt)-D-Pro-OMe ( <b>241</b> )	a	95
Fmoc-D-Phe-OH ( <b>242</b> )	D-Pro-OMe ( <b>223</b> )	Fmoc-D-Phe-D-Pro-OMe ( <b>243</b> )	a	79
Boc-D-Tyr(OBn)-OH ( <b>244</b> )	D-Pro-OMe ( <b>223</b> )	Boc-D-Tyr(OBn)-D-Pro-OMe ( <b>245</b> )	b	57
Fmoc-L-Arg(Pmc)-OH ( <b>246</b> )	L-Pro-OMe ( <b>226</b> )	Fmoc-L-Arg(Pmc)-L-Pro-OMe ( <b>247</b> )	a	70
Fmoc-L-Arg(Pmc)-OH ( <b>246</b> )	D-Pro-OMe ( <b>223</b> )	Fmoc-L-Arg(Pmc)-D-Pro-OMe ( <b>248</b> )	a	85
Fmoc-D-Arg(Pmc)-OH ( <b>249</b> )	L-Pro-OMe ( <b>226</b> )	Fmoc-D-Arg(Pmc)-L-Pro-OMe ( <b>250</b> )	a	83
Fmoc-D-Arg(Pmc)-OH ( <b>249</b> )	D-Pro-OMe ( <b>226</b> )	Fmoc-D-Arg(Pmc)-D-Pro-OMe ( <b>251</b> )	a	76

<sup>a</sup> Coupling reaction carried out with PyBOP, DIPEA.<sup>b</sup> Coupling reaction carried out with EDC.HCl, HOBt, DIPEA.

The compounds were isolated after purifications by column chromatography (MeOH/CH<sub>2</sub>Cl<sub>2</sub> gradient) giving the dipeptides in 59-95% yield. In the case of coupling Gly, L/D-Pro and D-Ser, it was difficult to remove the by-product from the PyBOP coupling, so EDC/HOBt was preferred.

For the cyclisation of the dipeptides, the linear protected dipeptides were treated with diethylamine in DMF for the removal of the Fmoc group. In the case of Boc-dipeptides (**231**), (**233**), (**235**), (**237**), (**239**) and (**245**), treatment with 4 M aq HCl in dioxane was carried out and the resulting hydrochloride salts were neutralized with DIPEA, before the cyclization (Scheme 94).



*Reagents and conditions:* (i) Et<sub>2</sub>NH/DMF or HCl-dioxane; (ii) cyclisation.

**Scheme 94.** Cyclisation of dipeptides to CI-4 analogues.

In an initial attempt to prepare the cyclic dipeptides cyclo(L-Pro-L-Pro) (**215**), cyclo(D-Pro-D-Pro) (**252**) and cyclo(Gly-D-Pro) (**253**), the corresponding linear dipeptides were prepared from the Fmoc amino acids (**224**), (**227**), (**229**). However, after the cyclization, the desired cyclic dipeptides were found to be contaminated by the by-product from the Fmoc deprotection, which was difficult to remove even on repeated column chromatography.

**Table 6.** Preparation of cyclic dipeptides

Comp.	Pro	Cyclo (Xaa-Pro)	Conditions	Yield%
(252)	D-Pro	(D-Pro-D-Pro)	MeOH <sup>(a)</sup> 65 °C, 24 h	33
(215)	L-Pro	(L-Pro-LPro)	MeOH <sup>(a)</sup> 65 °C, 24 h	33
(253)	D-Pro	(Gly-D-Pro)	MeOH <sup>(a)</sup> 65 °C, 24 h	45
(254)	D-Pro	(D-Ser(OBn)-D-Pro)	DMF <sup>(a)</sup> 65 °C, 24 h	46
(255)	L-Pro	(L-Ser(OBn)-L-Pro)	DMF <sup>(a)</sup> 65 °C, 24 h	53
(256)	D-Pro	(D-His(Trt)-D-Pro)	Toluene <sup>(b)</sup> 110 °C, 24 h	38
(213)	D-Pro	(D-Phe-D-Pro)	Toluene <sup>(b)</sup> 110 °C, 96 h	12
(257)	D-Pro	(D-Tyr(OBn)-D-Pro)	DMF <sup>(a)</sup> 80 °C, 24 h	64

<sup>a</sup> Deprotection of Boc with 4M HCl/dioxane, neutralize with DIPEA, before the cyclization.

<sup>b</sup> Deprotection of Fmoc with Et<sub>2</sub>NH/DMF before the cyclization.

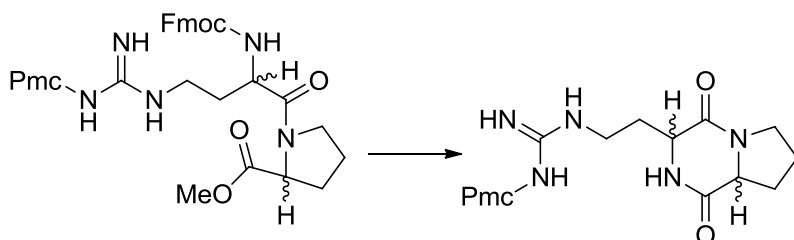
After the Fmoc deprotection of (241), (243), (247), (248), (250) and (251) using diethylamine/DMF solution, the cyclisation of the dipeptides proceeded in dilute methanol or toluene solution to give the cyclo(Xaa-Pro) in 12-89% yield. As noted above for Boc-dipeptides, DIPEA had to be used in order to neutralize the salt formed after the Boc deprotection with HCl/dioxane. For (213) and (256), the reaction did not proceed in MeOH at

65 °C, so toluene had to be used and heating at 110 °C, giving **(213)**, **(256)** in yields of 12% and 38% respectively.

As noted above, to achieve the cyclization of a dipeptide intermediate, the amide bond must assume the *cis*-configuration. This may be hindered by sterically bulky groups adjacent to C $\alpha$ , resulting in poor yields of cyclization. In these cases heating in high boiling solvents such as toluene or xylene may be needed to force the reaction to completion.

Although proline should favour a *cis* amide bond in the Xaa-Pro sequence desirable for formation of 2,5-DKPs, we did indeed obtain a poor yield in some cases. For the His(Trt) **(256)** and Phe **(213)** analogues, cyclisation was much less efficient, probably due to the size of the C $\beta$ -substituents.

For cyclo(L-Arg(Pmc)-D-Pro) **(259)**, carrying the reaction out at 65 °C gave two compounds with close retention times on HPLC, in 80% yield (see Figure 50). However, at room temperature, the reaction proceeded to give only **(259)** in 84% yield (Table 7).



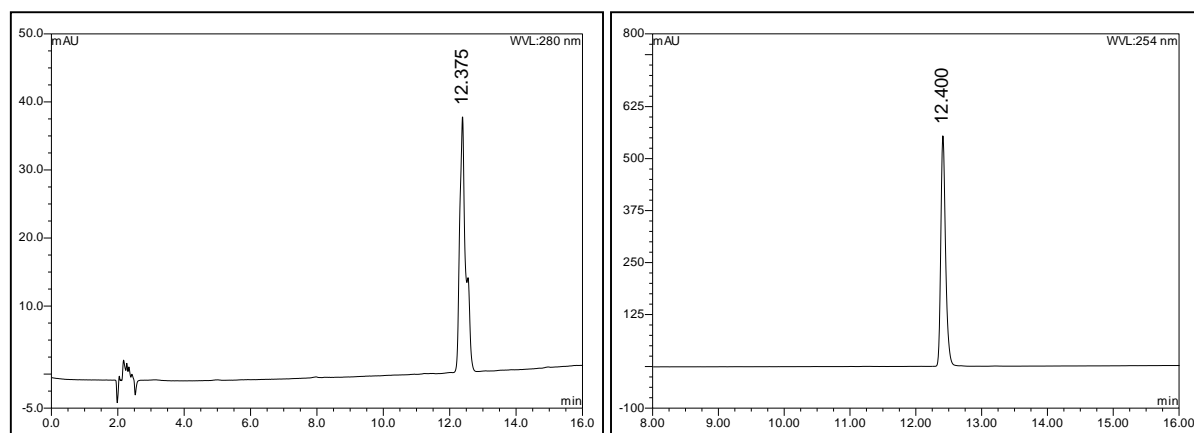
**Scheme 95.** Synthesis of cyclo(Arg(Pmc)-Pro) diastereoisomers.

**Table 7.** Synthesis of cyclo(Arg(Pmc)-Pro)

No	Arg(Pmc)	Pro	Yield %
<b>(258)</b>	L	L	89
<b>(259)</b>	L	D	84
<b>(260)</b>	D	L	87
<b>(261)</b>	D	D	77

Cyclisation of all four diastereoisomeric Arg(Pmc)-Pro-OMe derivatives at room temperature gave a single isomer as judged by HPLC and <sup>1</sup>H NMR. Compounds **(258)**/**(261)** and **(259)**/**(260)** could be clearly resolved by analytical HPLC. For the case of **(250)**, the product obtained from

reaction at 65°C shows evidence of other diastereoisomers both by HPLC (Figure 50) and <sup>1</sup>H NMR.



(a)

(b)

**Figure 50.** (a) HPLC trace of **(259)** plus epimer from the reaction at 65 °C in MeOH; (b) pure **(259)** from the reaction at rt.

#### Removal of Trt, OBn and Pmc groups

Deprotection of **(256)**, **(257)** and **(261)** was done under acidic conditions using TFA/TIPS/H<sub>2</sub>O (95/2.5/2.5, v/v/v). For the His derivative **(256)** more <sup>i</sup>Pr<sub>3</sub>SiH (2 eq) was added in order to quench the trityl cation generated.

**Table 8.** Deprotection of cyclic dipeptides

Comp.	Cyclo(Xaa-Pro)	Cyclo(Xaa-Pro)	Yields (%)
<b>(262)</b>	Cyclo (D-Arg(Pmc)-D-Pro) <b>(261)</b>	Cyclo (D-Arg-D-Pro) <b>(262)</b>	Qt yield
<b>(263)</b>	Cyclo(D-His(Trt)-D-Pro <b>(256)</b>	Cyclo(D-His(Trt)-D-Pro) <b>(263)</b>	Qt yield
<b>(264)</b>	Cyclo(D-Tyr(OBn)-D-Pro) <b>(257)</b>	Cyclo(D-Tyr-D-Pro) <b>(264)</b>	66

#### 5.4.5. Biological data

Selected cyclic dipeptides were evaluated against the bacterial chitinase, *SmChB1* from *S. marcescens*. This work was performed by Dr Gustav Vaaje-Kolstad (Norwegian University of Life Sciences, Ås, Norway).

Table 9 shows the results obtained with **(215)**, **(252)**, **(253)** and **(262)**, prepared in this study, and alongside IC<sub>50</sub> values reported in the literature for related cyclic dipeptides.

**Table 9.** IC<sub>50</sub> of the DKPs against *SmChiB1*

Compound	IC <sub>50</sub> (μM)
Cyclo (D-Arg(Pmc)-D-Pro) ( <b>262</b> )	63.5 ± 26
Cyclo (D-Pro-D-Pro) ( <b>252</b> )	136.9 ± 38
Cyclo(L-Pro-L-Pro) ( <b>215</b> )	469.4 ± 72
Cyclo(Gly-D-Pro) ( <b>253</b> )	1334
Cyclo(Gly-L-Pro) ( <b>266</b> )	5000 <sup>140</sup>
CI-4 Cyclo(L-Arg-D-Pro) ( <b>15</b> )	1200 <sup>140</sup>
Cyclo(L-Arg-L-Pro) ( <b>267</b> )	6300 <sup>140</sup>

The limited data available suggest that DKPs with the D-D template are the more potent compounds. **(262)** is more than 100-fold more potent than the natural product, CI-4, **(15)**.

Cyclo(L-Arg-L-Pro) (**267**) is a weaker inhibitor still compared to **(15)**. This confirms the important of D-configuration for the proline residue and also the potential for modifying the non-proline residue suggested by Houston *et al.*<sup>140</sup> This observation about the D-configuration is supported by the activity of cyclo(Gly-D-Pro) (**253**) which is significantly better than the L-Pro derivative. As expected, cyclo(D-Pro-D-Pro) (**252**) is a better inhibitor than its enantiomer cyclo(L-Pro-L-Pro) (**215**). Surprisingly however, although **(215)** is 4-fold weaker than **(252)**, it is a better inhibitor than **(253)** which does have a D-proline residue. This may suggest a new binding mode for **(215)** with SmChiB not seen with other DKPs,<sup>140</sup> which should be revealed by further X-ray studies.

Based on the initial data obtained, it seems possible that DKPs such as cyclo(D-Ser(OBn)-D-Pro) (**254**) cyclo(D-Tyr(OBn)-D-Pro) (**257**) and cyclo(D-His(Trt)-D-Pro) (**256**) will also be significantly more potent than CI-4 (**15**). This is based on the presence of a D-proline residues and a non-proline residue with a side chain bearing a bulky hydrophobic group, as in **(262)** which could potentially make hydrophobic interactions with the conserved tryptophan residues within the active site of the enzyme.

Data on further compounds should confirm these preliminary findings and provide the basis for design of other inhibitors based around DKP motifs with D-D configuration.



#### **5.4.6. Conclusion and future work**

A series of DKPs related to the natural product CI-4 has been synthesised. CI-4 has a very weak activity against the bacterial type chitinase *SmChiB* from *S. cerevisiae*. By changing the Pro configuration from L to D, compounds with promising activity have been obtained. Further compounds have been synthesized based on the D,D-template with the Arg residue replaced by a hydrophobic group to interact with the hydrophobic regions of chitinase active site. Biological data on these analogues should confirm our initial findings and provide the basis for the design of other inhibitors based around DKP motifs.

## 6. Overall conclusions

This project focused on the synthesis and development of peptide-derived chitinase inhibitors.

In Section 5, part 1, an improved synthesis of the cyclic pentapeptide inhibitor argifin was investigated. The synthesis was carried out by a combination of solution and solid phase methods in order to provide a synthetic route that might be suitable for scale-up. The linear peptide was synthesised by SPPS and was cyclised efficiently in solution. By choosing hydrogenolysable protecting groups for the side chains of Asp and Arg residues, it was possible to avoid a final deprotection of the final cyclic peptide under acidic conditions, which has been shown to lead to the generation of aspartimide products. The final yield of the natural product from the linear peptide was 19%, which compares very favourably with previously reported syntheses. Importantly, only one HPLC purification was required at the final step of the synthesis, which should allow the synthetic route to be easily applied for the preparation of mg-quantities of the natural product.

New methods have also been studied for the preparation of the guanyljurea side chain unit of argifin and related chitinase inhibitors. In Section 5, part 1, the synthesis of reagents for the direct conversion of amines to guanyljureas were also explored. The direct conversion of a bis-benzyloxycarbonyl-protected arginine residue, Arg(Z)<sub>2</sub> to the corresponding guanyljurea was first investigated, based on the observation that such a transformation could be successfully achieved by reaction of simple guanidine derivatives with methylamine. The result of such an aminolysis reaction was inconclusive when applied to a protected cyclic pentapeptide related to argifin and when similar reactions were investigated with shorter peptides containing the benzyloxycarbonyl protecting group, there was no success. Therefore we looked at building blocks that could be converted to potential guanidinating agents, which could then be applied for the conversion of an Orn side chain into Arg(MC).

A range of Boc- and Z-protected guanyljureas were obtained by reaction of bis-Boc and bis-Z guanidines with methylamine, *N*-methylbenzylamine and 4-methoxy-*N*-methylbenzylamine in poor to good yields. The aim was to generate guanidinyllating agents related to previously reported bis-urethane-trifluoromethylsulfonamides and tris-Boc/Z compounds. One of the guanyljureas prepared was successfully converted to a bis-Boc derivative, which, in the future, could be used to test the reactivity of compounds of this type with different amines.

In Section 5, parts 2 and 3, the synthesis of a key component of the cyclic peptide banyasin A was investigated. Banyasin A is a natural product which contains the Arg(MC)MePhe dipeptide motif of argifin, which therefore suggests that it may be an interesting chitinase inhibitor. It also contains the novel amino acid, Amoa (3-amino-2-methyl-5-*E*-octenoic acid), in which the stereochemical configurations at C-2 and C-3 are unresolved. In these sections of the work, the synthesis of four diastereoisomers of Amoa was studied in some detail.

A potential approach to the novel amino acid Amoa and its diastereoisomers was first studied based on the Crimmins modification of the Evans aldol reaction. Although aldol derivatives were successfully prepared using this Ti-based chemistry, introduction of a nitrogen function by nucleophilic displacement with azide and Mitsunobu chemistry proved to be problematic. The desired products were only obtained in low yields and significant amounts of eliminated products were observed. It was therefore decided to pursue an alternative chiral pool approach which would involve introducing the C-2 methyl group of Amoa into an enantiomerically pure  $\alpha$ -amino derivative.

L-Aspartic acid was chosen as the chiral starting material with the key nitrogen function already in place. The combination of different protecting groups on the nitrogen and  $\alpha$  and  $\beta$ - carboxylic acid groups were found to be important for the diastereoselectivity of the methylation reaction, in accordance with the literature. The best results were obtained using distilled reagents, iodomethane and lithium hexamethyldisilazane, which gave the desired *anti*  $\beta$ -methyl aspartate in 48% yield. Surprisingly, the use of HMPA in the alkylation reaction did not improve the diastereoselectivity as has been previously reported but instead led to the formation of both *N*- and C-alkylated products.

Two routes were considered for the conversion of the selectively protected  $\beta$ -methyl aspartate to Amoa. The key transformation to be achieved was a homologation reaction at the  $\alpha$ -acid group. Selective reduction of the  $\alpha$ -acid to the aldehyde *via* the thiol ester or Weinreb amide was first attempted – the aldehyde could then be reacted with a suitable phosphorane in a Wittig-type reaction. Although the Weinreb amide could be selectively reduced with DIBAL-H, it was not possible to separate the desired aldehyde from unchanged starting material. Reduction of the thiol ester using triethylsilane and a Pd catalyst was also unsuccessful, as parallel reduction of the allyl ester group could not be avoided. Finally, a successful and selective route was devised, *via* Arndt-Eistert chemistry. This led to the desired homologated  $\beta$ -amino acid

derivative, (3*R*,4*R*)-3-(((benzyloxy)carbonyl)amino)-4-methyl-5-oxo-5-allyloxypentanoic acid in 27% yield over 8 steps from L-aspartic acid.

With this key intermediate in hand, the synthesis of Amoa may be completed as follows. Conversion of the new carboxylic function to the corresponding aldehyde maybe achieved by reduction to the primary alcohol and then selective re-oxidation under Swern conditions. The alkene function of Amoa might then be introduced by *E*-selective Julia-Kocienski olefination to give the protected Amoa (2*R*, 3*R*)-diastereoisomer. Since we have observed that changing the protecting groups on the L-aspartic acid starting material leads to differing ratios of separable diastereoisomers in the methylation reaction with iodomethane, it should be possible to obtain readily the other isomer to generate the (2*S*, 3*R*) Amoa diastereoisomer. Similarly, by changing to suitable protected D-aspartic acid starting materials, it should also be possible to generate the two other (2*S*, 3*S*) and (2*R*, 3*S*) diastereoisomers of Amoa. With all four diastereoisomers of Amoa in hand, it will then be possible to apply these intermediates for the synthesis of banyasin A itself and identify the one present in the natural product synthesised.

In Section 5, part 4, several analogues of CI-4 cyclo(L-Arg-D-Pro), were synthesized, with a D-D template. It is known from the literature that cyclo(L-Arg-L-Pro), a diastereoisomer of CI-4, is a weaker inhibitor than CI-4 itself. X-ray data show that changing the stereochemical configuration at the proline residue changes the binding orientation of the DKP in the active site of a typical bacterial chitinase, while the arginine residue does not make any significant contacts with the enzyme in CI-4. It has been suggested therefore that the arginine residue might be replaced with alternatives that present a hydrophobic group that can interact with conserved hydrophobic tryptophan residues in the chitinase active site.

We chose to prepare DKPs with hydrophobic amino acid side chains, or side chains with bulky protecting groups such as Ser(OBn), Tyr(OBn), His(Trt) and Phe in order to favour these interactions. DKPs with an all-D stereochemical configurations were synthesized in yields of 12% to 89%. Preliminary biological data obtained with the bacterial chitinase SmChiB showed that some of these compounds, were as expected, more potent than CI-4 and significantly more potent than related DKPs with all-L configuration in the literature.

Based on the results obtained in this work, further studies on CI-4 analogues may now provide a starting point for the development of new small molecule chitinase inhibitors.

## 7. Experimental

### 7.1 General

Chemical reagents were purchased from Sigma, Aldrich, Fluka, Acros, Lancaster and Novabiochem. Anhydrous  $\text{CH}_2\text{Cl}_2$  was obtained by distillation over calcium hydride, anhydrous THF and  $\text{Et}_2\text{O}$  over sodium/benzophenone. All other solvents were purchased from Fisher Scientific. DMF peptide grade was purchased from Rathburn Chemicals Ltd, Walkerburn, Scotland.

Analytical TLC was performed using silica gel 60 F<sub>254</sub> pre-coated on aluminium sheets (0.25 mm thickness). They were viewed using UV light of wavelength 254 nm and stained with iodine, ninhydrin, and phosphomolybdic acid. Column chromatography was performed on silica gel 60 (35-70 micron) from Fisher Scientific.

Melting points were recorded on a Reichert-Jung Kofler block heated-stage microscope and are uncorrected.

Optical rotations were measured at ambient temperature using an Optical Activity Ltd. AA-10 polarimeter in a cell volume of 5 mL and specific rotations are given in  $10^{-1}$  deg. mL g<sup>-1</sup>.

Infrared spectra were recorded on a Perkin-Elmer RXI FT-IR system and values are given in  $\text{cm}^{-1}$ .

<sup>1</sup>H NMR spectra were obtained on JEOL Eclipse (270 MHz), Varian Mercury VX (400 MHz), Bruker Avance III (400 MHz) or Bruker Avance III (500 MHz) spectrometers. <sup>13</sup>C NMR spectra were obtained on JEOL Eclipse (67.9 MHz), Varian Mercury VX (100 MHz) Bruker Avance III (100 MHz) or Bruker Avance III (125 MHz) spectrometers. The chemical shifts are recorded in parts per million (ppm) with reference to tetramethylsilane. The coupling constants *J* are quoted in Hz. The multiplicities are assigned as a singlet (s), doublet (d), triplet (t), quartet (q), quintuplet (qn), doublet of doublets (dd), triplet of doublets (td), broad (br), and multiplet (m).

Mass Spectrometry was carried out on a micrOTOF<sup>TM</sup> from Bruker Daltonics (Bremen, Germany), using an electrospray source (ESI-TOF). This is a Time-of-Flight mass spectrometer

coupled with an electrospray source (ESI-TOF). This instrument can be used to measure accurate mass to 5 ppm externally calibrated and 2 ppm internally calibrated. Samples are introduced by injection using an autosampler system. The instrument is calibrated using a sodium formate solution, which is applicable to both positive- and negative-ionisation modes. These samples were analysed under standard conditions for small molecules in positive electrospray ionisation mode. The instrument acquires both accurate mass and true isotope patterns, therefore both of these dimensions of information are used to help determine or confirm the molecular formula. Ions are most often present as protonated or sodiated molecules. Data were processed using external calibration with the Bruker Daltonics software, DataAnalysis<sup>TM</sup> as part of the overall hardware control software, Compass 1.1<sup>TM</sup>.

Analytical RP-HPLC was performed on a Dionex UltiMate 3000 HPLC system equipped with a Phenomenex Gemini 5 $\mu$ m C-18 (150 x 4.6 mm) column with a flow rate of 1 mL/min. Preparative RP-HPLC was performed on a Dionex HPLC system equipped with a Phenomenex Gemini 5 $\mu$ m C-18 (250 x 30 mm) column with a flow rate of 22.5 mL/min. Mobile phase A was 0.1% TFA in water, mobile phase B was 0.1% TFA in acetonitrile.

Gradient 1 was  $T = 0$  min,  $B = 5\%$ ;  $T = 10$  min,  $B = 95\%$ ;  $T = 15$  min,  $B = 95\%$ ;  $T = 15.1$  min,  $B = 5\%$ ;  $T = 18.1$  min,  $B = 5\%$ .

Gradient 2 was  $T = 0$  min,  $B = 5\%$ ;  $T = 20$  min,  $B = 40\%$ ;  $T = 22$  min,  $B = 95\%$ ;  $T = 27$  min,  $B = 95\%$ ;  $T = 27.1$  min,  $B = 5\%$ ;  $T = 32$  min,  $B = 5\%$ .

Gradient 3 was  $T = 0$  min,  $B = 5\%$ ;  $T = 30$  min,  $B = 12\%$ ;  $T = 32$  min,  $B = 95\%$ ;  $T = 37$  min,  $B = 95\%$ ;  $T = 38$  min,  $B = 5\%$ ;  $T = 42$  min,  $B = 5\%$ ;  $T = 42.1$  min,  $B = 5\%$ .

Qualitative tests for the presence or absence of free primary amino groups during deprotection or coupling in SPPS were carried out with the Kaiser method.<sup>250</sup> The beads are treated with ninhydrin in ethanol, phenol in ethanol, and potassium cyanide, then heated. A positive result is indicated by the beads turning dark blue. For secondary amines, the chloranil test was used.<sup>251</sup> The beads are treated with acetaldehyde in DMF, and p-chloranil in DMF. The presence of secondary amines is indicated by the beads turning to bright orange/red.

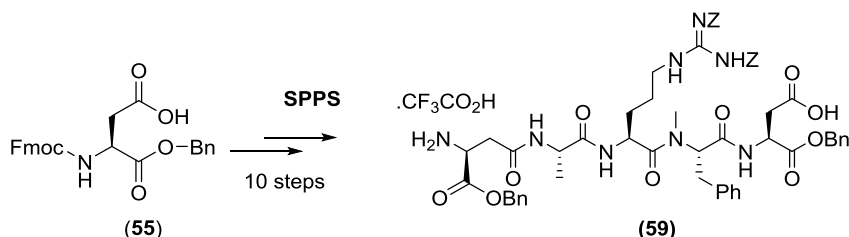
Enzyme activities of ChiB in the presence of the different cyclic dipeptides were determined using 4-methylumbelliferyl- $\alpha$ -D-*N,N*-diacetylchitobioside as a fluorogenic substrate in a standard assay.

Reaction mixtures consisted of purified ChiB (2.75 nM), substrate (20  $\mu$ M), BSA (0.1 mg/mL), and inhibitor (0-10 mM) in 50  $\mu$ L citrate/phosphate buffer pH 6.3. The mixtures were incubated for 10 min at 37 °C (product formation is known to be linear over time under these conditions) and the reaction was stopped by addition of 0.2M Na<sub>2</sub>CO<sub>3</sub> (1.95 mL). The amount of liberated 4-methylumbelliferone (4-MU) was determined using a DyNA 200 fluorimeter (Hoefer Pharmacia Biotech, San Francisco, CA). Each measurement was performed in triplicate, yielding standard deviations that in all cases were below 5% of the average. IC<sub>50</sub> values were estimated from plots of relative activity versus inhibitor concentration.

## 7.2. Approaches to arginin and related peptides

### 7.2.1. Improved synthesis of arginin by use of hydrogenolysable protecting groups.

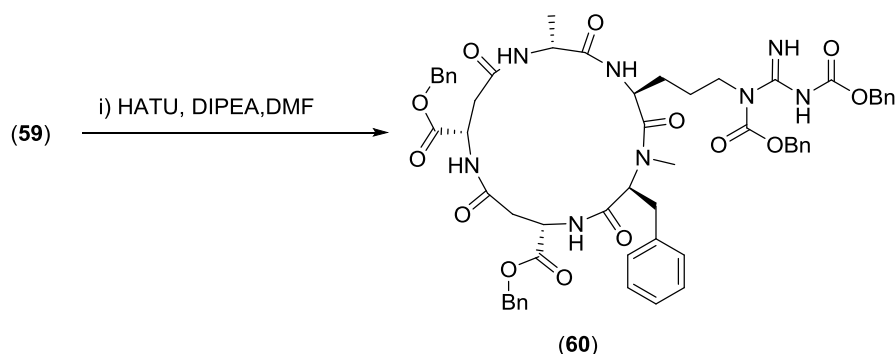
#### H-βAsp(OBn)-D-Ala-Arg(Z)<sub>2</sub>-MePhe-Asp-OBn (**59**)



Synthesis was carried out manually by Fmoc SPPS. Wang resin (0.354 g nominal loading 1.21 mmol/g, 0.390 mmol) was pre-swollen in dry CH<sub>2</sub>Cl<sub>2</sub> (7 mL) for 30 min. The loading of the resin was achieved as follows: Fmoc-Asp-OBn (0.523 g, 1.17 mmol) was dissolved in dry CH<sub>2</sub>Cl<sub>2</sub> (4 mL) and DMF (3 mL), DIC (0.75 g, 0.59 mmol) in dry CH<sub>2</sub>Cl<sub>2</sub> (3 mL) was added and the mixture was stirred for 20 min at 0° C. All the solvents were evaporated and the residue of Fmoc-Asp anhydride was dissolved in DMF (7 mL). The resin (0.354 g, 0.390 mmol) was suspended in anhydrous CH<sub>2</sub>Cl<sub>2</sub> (5 mL) and was treated with DMAP (10.2 mg, 78.0 μmol) plus the solution of Fmoc-Asp anhydride and stirring was continued at room temperature for 90 min. The resin was washed with DMF (4 x 7 mL), CH<sub>2</sub>Cl<sub>2</sub> (4 x 7 mL), MeOH (4 x 7 mL), Et<sub>2</sub>O (4 x 7 mL). Fmoc deprotection was achieved by treatment with piperidine/DMF (1:4, v/v) (3 x 7 mL), for 4 x 3 min. Peptide couplings were performed based on the nominal loading, using Fmoc-amino acid (2 eq), PyBOP (1.9 eq) and DIPEA (8 eq) in peptide grade DMF (5 mL) for 90 min, except for the case of coupling to MePhe when Fmoc-Arg(Z)<sub>2</sub>-OH (2 eq), HATU (2 eq) and DIPEA (4 eq) in DMF peptide grade (5 mL) for 150 min was used. The last Asp derivative coupled was Boc-Asp-OBn. The resin was washed with DMF (4 x 7 mL), CH<sub>2</sub>Cl<sub>2</sub> (4 x 7 mL), MeOH (4 x 7 mL), Et<sub>2</sub>O (4 x 7 mL). Cleavage from the resin and the side chain deprotection were achieved by treatment with TFA/TIPS/H<sub>2</sub>O (95:2.5:2.5, v/v/v) (10 mL). All the solvents were evaporated under vacuum and the residue was dissolved in TFA and cold Et<sub>2</sub>O was added. The precipitated white solid was isolated by centrifugation, to give (**59**) as a white solid (371 mg, 80% yield); **RP-HPLC** (analytical system, gradient 1) R<sub>t</sub>= 9.1 min), [found (ES<sup>+</sup>) 1085.4630 [M+H]<sup>+</sup>, C<sub>57</sub>H<sub>65</sub>N<sub>8</sub>O<sub>14</sub> requires 1085.4615].

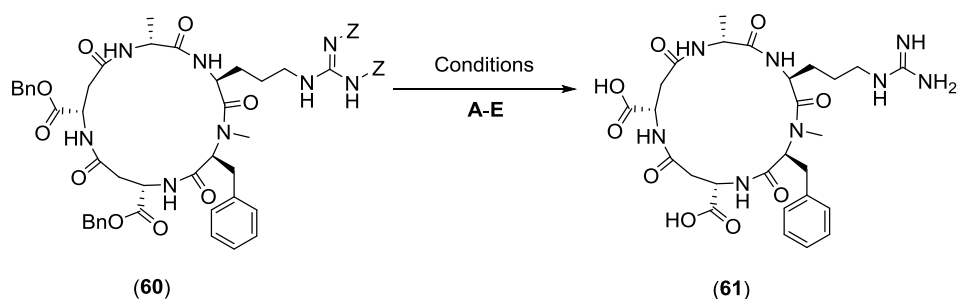


### Cyclo (βAsp(OBn)-D-Ala-Arg(Z<sub>2</sub>)-MePhe-β Asp(OBn)) (60)



Compound (**59**) (371 mg, 0.310 mmol), and HATU (118 mg, 0.310 mmol) were dissolved in anhydrous  $\text{CH}_2\text{Cl}_2$  (20 mL), and the solution was adjusted to pH 9 by addition of DIPEA (520  $\mu\text{L}$ , 1.43 mmol) and stirred for 48 h at room temperature. The reaction mixture was concentrated under vacuum and the residue dissolved in EtOAc (20 mL) and extracted with 5% aq. citric acid (2 x 40 mL), sat. aq.  $\text{NaHCO}_3$  (2 x 40 mL) and brine (2 x 40 mL). The organic extract was filtered and dried over  $\text{MgSO}_4$  to give a yellow oil (192 mg). The HPLC of the crude cyclic pentapeptide was run and showed two peaks, **RP-HPLC** (analytical system, gradient 1)  $R_t = 8.6$  min, and  $R_t = 10.8$  min. The crude material was precipitated from  $\text{CH}_2\text{Cl}_2$ /petroleum ether, to give (**60**) as a pale yellow solid (182 mg, 55%); **RP-HPLC** (analytical system, gradient 1)  $R_t = 10.8$  min. [found (ES<sup>+</sup>) 1089.4381  $[\text{M}+\text{Na}]^+$ ,  $\text{C}_{57}\text{H}_{63}\text{N}_8\text{O}_{13}\text{Na}$  requires 1089.4329].

### Cyclo(βAsp-D-Ala-Arg-MePhe-βAsp) (61)

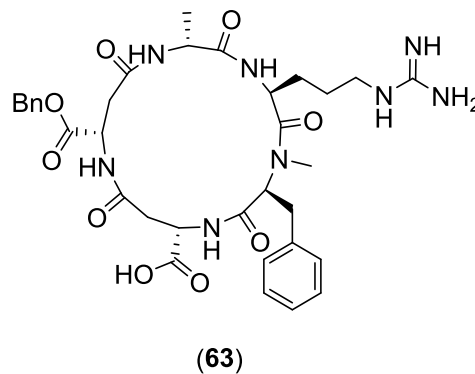
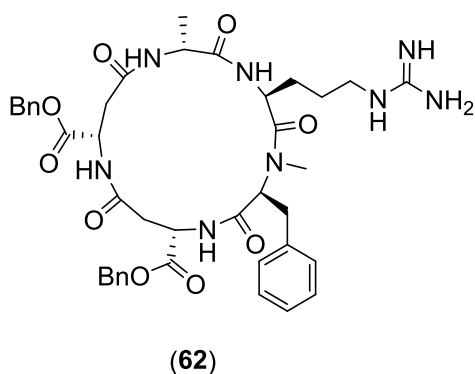


**A:**  $\text{H}_2$ ,  $\text{Pd}(\text{OH})_2/\text{C}$ ,  $\text{AcOH}/i\text{PrOH}$ , rt, 1 h; **B:**  $\text{H}_2$ , Pd black,  $\text{AcOH}/i\text{PrOH}$ , rt, 18 h; **C:**  $\text{H}_2$ ,  $\text{Pd}(\text{OH})_2/\text{C}$ ,  $\text{AcOH}/i\text{PrOH}$ , rt, 16 h; **D:**  $\text{H}_2$ ,  $\text{Pd}(\text{OH})_2/\text{C}$ ,  $\text{AcOH}/i\text{PrOH}$ , rt, 18 h; **E:**  $\text{H}_2$  (4 Bar, hydrogenator),  $\text{Pd}(\text{OH})_2/\text{C}$ ,  $\text{AcOH}$ , rt, 72 h.

**Method A:** Compound (**60**) (40.0 mg, 37.0  $\mu\text{mol}$ ), was dissolved in  $\text{AcOH}/i\text{PrOH}$  (20 mL, 1:1 v/v), the solution was degassed and purged three times with nitrogen. The solution was treated

with  $\text{Pd}(\text{OH})_2/\text{C}$  (40 mg). The reaction was degassed three times with nitrogen before treating with  $\text{H}_2$  (1 atm) and stirring continued at room temperature for 1 h. The reaction mixture was monitored by TLC (10%  $\text{MeOH}/\text{CH}_2\text{Cl}_2$ ), until consumption of the starting material was observed. The reaction mixture was filtered through Celite, then coevaporated with  $\text{Et}_2\text{O}$ , then  $\text{MeOH}$  and finally with toluene to give a pale white solid (31.4 mg). HPLC indicated the disappearance of the starting material and formation of a new compound, **RP-HPLC** (analytical system, gradient 1)  $R_t = 9.9$  min.

Mass spectrometry indicated two compounds, corresponding respectively to the deprotection of the arginine residue (**62**), [found ( $\text{ES}^+$ ) 799.3774  $[\text{M}+\text{H}]^+$ ,  $\text{C}_{41}\text{H}_{50}\text{N}_8\text{O}_9$  requires 799.3740] and arginine deprotection plus removal of the one of two Bn group on the aspartic acid residues (**63**), [found ( $\text{ES}^+$ ) 731.3123  $[\text{M}+\text{Na}]^+$ ,  $\text{C}_{34}\text{H}_{44}\text{N}_8\text{O}_9\text{Na}$  requires 731.3133].



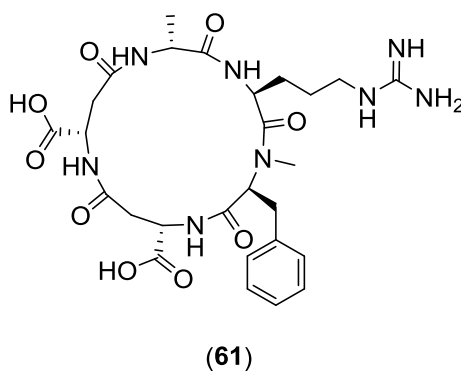
**Method B:** The mixture of those two cyclic pentapeptides (**62**) and (**63**) from method **A** was again submitted to hydrogenolysis using  $\text{Pd}(\text{OH})_2/\text{C}$  (31 mg). The reaction was run for 18 h at room temperature but no further change was observed by RP-HPLC.

**Method C:** Same sample from Method B. A mixture of partially deprotected cyclic pentapeptide (31 mg) was dissolved in  $\text{AcOH}/i\text{PrOH}$  (20 mL, 1:1 v/v) and the solution was degassed and purged three times with nitrogen. The reaction mixture was then treated with Pd black (31 mg), and was degassed three times with nitrogen before treating with  $\text{H}_2$  (1 atm), and stirring continued at room temperature, for 16 h. RP-HPLC showed no further change.

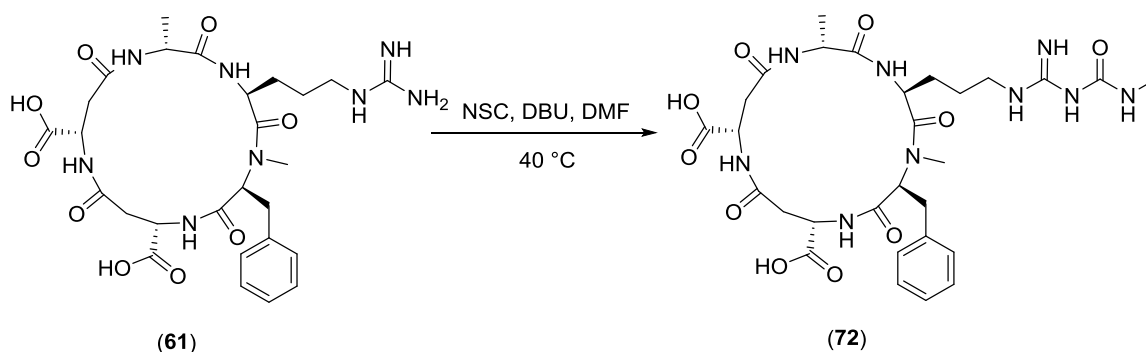
**Method D:** Compound (**60**) (20.0 mg, 19.0  $\mu\text{mol}$ ) was dissolved in  $\text{AcOH}/i\text{PrOH}$  (3 mL, 1:1 v/v), the solution was degassed and purged three times with nitrogen. The reaction mixture was then treated with a fresh sample of  $\text{Pd}(\text{OH})_2/\text{C}$  (20 mg) and was degassed and purged three times with nitrogen before treating with  $\text{H}_2$  (1 bar) and stirring at room temperature for 18 h.

The reaction mixture was monitored by TLC (MeOH/CH<sub>2</sub>Cl<sub>2</sub>), which showed the consumption of the starting material. The reaction was filtered through Celite, then coevaporated with Et<sub>2</sub>O then MeOH and finally toluene to give a pale white solid (31.4 mg); **RP-HPLC** (analytical system, gradient 1) R<sub>t</sub> = 9.9 min.

**Method E:** Compound (**60**) (20.0 mg, 19.0  $\mu\text{mol}$ ) was dissolved in AcOH (7 mL), the solution was degassed and purged three times with nitrogen. The reaction mixture was then treated with  $\text{Pd}(\text{OH})_2/\text{C}$  (60 mg) and hydrogenated at 4 bar pressure and stirred at room temperature for 72 h. HPLC indicated the disappearance of the starting material and formation of a new compound, **RP-HPLC** (analytical system, gradient 1)  $R_t = 4.2$  min). This gave the fully deprotected cyclic pentapeptide (**61**) (30 mg), which was used directly in the subsequent acylation step. [found (ES+) 619.2874  $[\text{M}+\text{Na}]^+$ ,  $\text{C}_{27}\text{H}_{39}\text{N}_8\text{O}_9\text{Na}$  requires 619.2835].



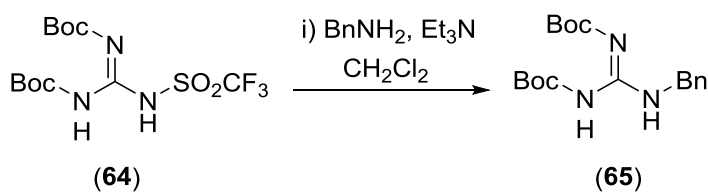
**Cyclo( $\beta$ Asp-D-Ala-Arg(MC)-MePhe- $\beta$ Asp)<sup>161</sup> (72)**



A solution of **(61)** (30.0 mg, 19.0  $\mu$ mol) in DMF (0.8 mL) and DBU (24.0  $\mu$ L, 0.162 mmol) was stirred at 40 °C and was treated with *N*-succinimidyl-*N*-methylcarbamate (14.0 mg, 0.810  $\mu$ mol) in DMF (0.4 mL). The reaction mixture was stirred at 40°C for 48 h. The acylated compound was then purified by preparative HPLC, to give **(72)** as a solid (3.5 mg, 19%, from **(60)**); **RP-HPLC** (analytical system, gradient 1)  $R_t$  = 6.8 min. [found (ES+) 676.3046 [M+H]<sup>+</sup>, C<sub>29</sub>H<sub>42</sub>N<sub>9</sub>O<sub>10</sub> requires 676.3054].

### 7.2.2. Approaches to alternative reagents for the direct conversion of Orn to Arg(MC)

***N,N'*-di-Boc-*N''*-benzylguanidine (65)**<sup>161</sup>

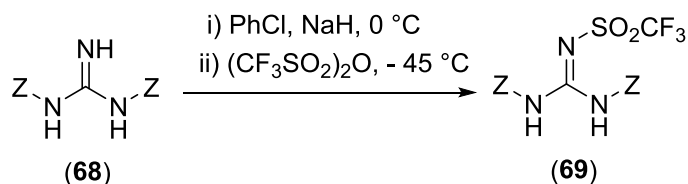


A stirred solution of benzylamine (53.0 mg, 0.50 mmol) and 1,3-di-Boc-2-(trifluoromethanesulfonyl)guanidine (**64**) (176 mg, 0.450 mmol) in CH<sub>2</sub>Cl<sub>2</sub> (2 mL) was treated with triethylamine (70  $\mu$ L, 0.50 mmol) and the reaction mixture was stirred at room temperature for 30 min after which time the starting material was consumed, as shown by TLC (10% EtOAc/petroleum ether). The reaction mixture was diluted into CH<sub>2</sub>Cl<sub>2</sub> (3 mL), and was washed with 2M aq. sodium bisulfate (10 mL), sat. aq. sodium carbonate (10 mL) and brine (10 mL). The organic extract was dried, filtered and the solvent was evaporated to give (**64**), as a white solid (114 mg, 72%); **Mp** 121-122  $^{\circ}$ C (lit.<sup>161</sup> 126-127  $^{\circ}$ C); **IR**  $\nu_{\text{max}}$  (KBr disc ) 3442,



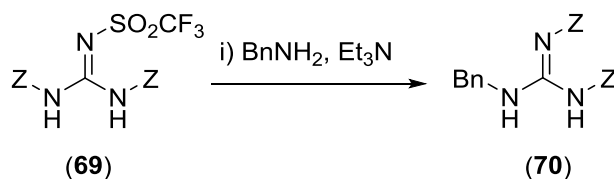
vigorous stirring over 45 min. Stirring was continued at 0 °C - 5° C overnight. The reaction was checked by TLC (1% MeOH/CH<sub>2</sub>Cl<sub>2</sub>), showing disappearance of the starting material and the reaction mixture was diluted in CH<sub>2</sub>Cl<sub>2</sub> (100 mL). The aqueous phase was extracted with CH<sub>2</sub>Cl<sub>2</sub> (100 mL). The organic fractions were washed with water (2 x 100 mL), were dried, filtered and the solvent was evaporated to give a white solid. The crude product was recrystallised from CH<sub>2</sub>Cl<sub>2</sub>/petroleum ether, to afford (**68**) as a white solid (7.77 g, 59%); **Mp** 148-150 °C (lit, <sup>164</sup> 149-150 °C); **IR**  $\nu_{\text{max}}$  (KBr disc) 3401, 3240 (NH), 3034, 2953 (CH), 1734, 1622, 1561 (C=O), 1443, 1384 (Ar), 1294, 1223 (CO), 755 (CH Ar); **<sup>1</sup>H NMR** (270 MHz, DMSO-d<sub>6</sub>)  $\delta$  5.16 (4H, s, 2 x CH<sub>2</sub>, PhCH<sub>2</sub>), 7.34-7.36 (10H, m, 2xPh), 8.68 (1H, br, NH), 10.89 (1H, br, NH).

***N,N'*-di-*Z*-*N''*-trifluoromethanesulfonylguanidine (69)**



A suspension of **(68)** (0.83 g, 2.5 mmol) in anhydrous chlorobenzene (25 mL) was treated with 60% sodium hydride (0.2 g, 8.3 mmol assumed) at 0 °C under a nitrogen atmosphere. After being stirred for 1 h at 0 °C, the mixture was cooled to -45 °C. Triflic anhydride (0.42 mL, 5.0 mmol) was added and the reaction mixture was allowed to warm to room temperature and stir overnight. The reaction was checked by TLC, *R<sub>f</sub>* 0.81 (5% Et<sub>2</sub>O/CH<sub>2</sub>Cl<sub>2</sub>), showing disappearance of the starting material. The solvent was evaporated, and the residue was dissolved in a mixture of EtOAc (50 mL) and 2M aq. sodium bisulfate (15 mL). The organic layer was washed with water (50 mL) and brine (50 mL), was dried, filtered and the solvent was evaporated to give a colourless oil. This residue was co-evaporated with toluene (3 x 100 mL) to remove traces of chlorobenzene and the solvent was evaporated, to afford **(69)** as a white solid (0.98 g, 84%); **Mp** 70-72 °C (lit, <sup>164</sup> 74-75 °C); <sup>1</sup>H NMR (270 MHz, CDCl<sub>3</sub>) δ 5.21 (4H, s, 2 x PhCH<sub>2</sub>), 7.24-7.26 (10H, m, 2 x Ph), 10.35 (1H, br, NH).

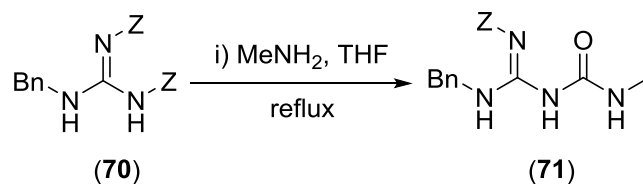
***N''*-benzyl-*N,N'*-di-*Z*-guanidine (70)<sup>164</sup>**



A stirred solution of **(69)** (230 mg, 0.500 mmol) and benzylamine (60 µL, 0.55 mmol) in CH<sub>2</sub>Cl<sub>2</sub> (8 mL) was treated with triethylamine (77 µL, 0.55 mmol) and the reaction mixture was stirred at room temperature. The reaction was continued until the starting material was consumed, as shown by TLC (20% Et<sub>2</sub>O/petroleum ether). The reaction mixture was washed with 2M aq. sodium bisulfate (10 mL), sat. aq. sodium carbonate (10 mL) and brine (10 mL). The organic extract was dried, filtered and evaporated to give **(70)** as a yellow solid (169 mg, 81%); **Mp** 150-152 °C<sup>161</sup>; <sup>1</sup>H NMR (270 MHz, CDCl<sub>3</sub>) δ 4.63 (2H, d, *J* 5.5 Hz, NHCH<sub>2</sub>Ph), 5.14 (2H,

s, OCH<sub>2</sub>Ph), 5.16 (2H, s, OCH<sub>2</sub>Ph), 7.32-7.36, (15H, m, 3 x Ph), 8.72 (1H, br, NH), 11.79 (1H, br, NH).

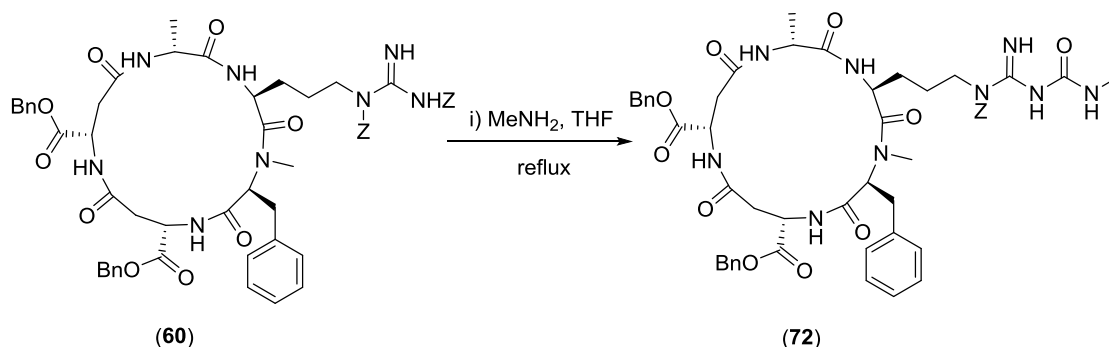
**(*E*)-Benzyl (benzylamino) (3-methylureido) methylenecarbamate (71)**



A solution of **(70)** (100 mg, 0.239 mmol) in CH<sub>2</sub>Cl<sub>2</sub> (5 mL) was treated with a solution of 2M methylamine in THF (0.60 mL, 1.20 mmol). The reaction mixture was stirred at reflux overnight. The solvent was evaporated to give a colourless oil. The residue was pre-adsorbed onto silica using CH<sub>2</sub>Cl<sub>2</sub> as a solvent and was purified by column chromatography, (EtOAc /petroleum ether gradient) to give **(71)** as white solid (80 mg, 98%); **Mp** 162-165 °C; **<sup>1</sup>H NMR** (270 MHz, DMSO-d<sub>6</sub>) δ 2.51 (3H, d, *J* 5.5 Hz, NHCH<sub>3</sub>), 4.53 (2H, d, *J* 5.5 Hz, PhCH<sub>2</sub>), 5.03 (2H, s, CH<sub>2</sub>, OCH<sub>2</sub>Ph) 7.30-7.32 (10H, m, 2 x Ph), 8.15 (1H, br, NH), 9.64 (1H, br, NH), 11.04 (1H, s, NH); **<sup>13</sup>C NMR** (CDCl<sub>3</sub>, 101 MHz) δ 28.34 (CH<sub>3</sub>), 45.12 (CH<sub>2</sub>Ph), 53.40 (CH<sub>2</sub>Ph), 127.41, 127.73, 128.06, 128.64, 128.75 (CH Ar), 137.81 (C quaternary), 152.86 (C=N), 163.28 (C=O); [found (ES<sup>+</sup>) 341.1592 [M+H], C<sub>18</sub>H<sub>21</sub>N<sub>4</sub>O<sub>3</sub> requires 341.1613].

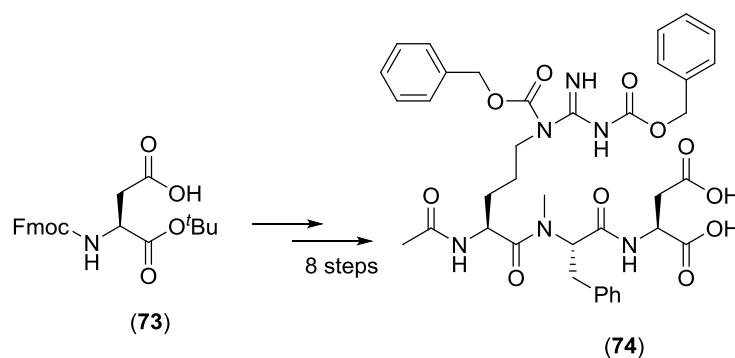


### Attempted conversion of cyclic peptide (60) into guanylurea derivative (72)



A solution of (60) (48 mg, 45  $\mu$ mol) was dissolved in THF (2 mL) and was then treated with a 2M solution of methylamine in THF (113  $\mu$ L, 0.225 mmol). The reaction mixture was stirred at 65 °C overnight. The reaction was followed by HPLC and 5 eq of MeNH<sub>2</sub> were added each day during 5 days in order to complete the reaction. After running the reaction for 24 h, a new peak appeared at  $R_t$  = 10.3 min. After 48 h and 72 h the new peak had increased compared to the starting material  $R_t$  = 10.7 min. After running the reaction for 5 days the HPLC showed the relative area of the peak at  $R_t$  10.3 min was significantly greater compared to the starting material. The solvents were evaporated, to give crude material as an oil (20 mg). Mass spectrometry showed the presence of starting material plus a new component corresponding to the incorporation of 2 eq of methylamine with loss of 2 benzyloxy groups [found (ES<sup>+</sup>) 933.4193 [M+Na]<sup>+</sup> C<sub>45</sub>H<sub>56</sub>N<sub>10</sub>O<sub>11</sub>Na, requires 933.4200].

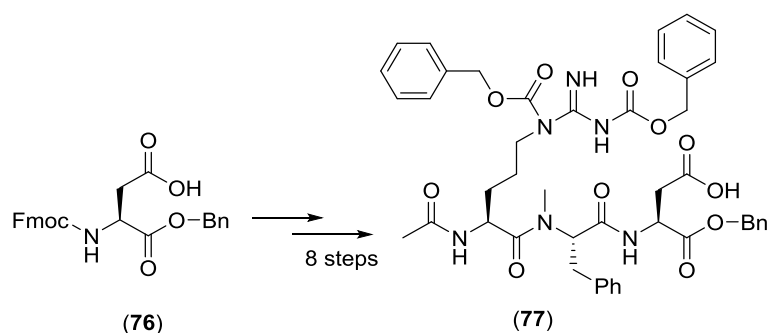
### Ac-Arg(Z)<sub>2</sub>-MePhe-Asp-OH (74)



Synthesis was carried out manually, by Fmoc SPPS. The 2-chlorotrityl resin (0.20 g, 0.22 mmol) was pre-swollen in dry CH<sub>2</sub>Cl<sub>2</sub> (7 mL) for 30 min. The resin was added to a solution of

Fmoc-Asp-O<sup>t</sup>Bu (**73**) (0.32 g, 0.78 mmol), and DIPEA (1.1 mL, 6.2 mmol) in anhydrous CH<sub>2</sub>Cl<sub>2</sub> (5 mL), with stirring continued at room temperature for 90 min. The resin was washed with DMF (4 x 7 mL), CH<sub>2</sub>Cl<sub>2</sub> (4 x 7 mL), MeOH (4 x 7 mL), Et<sub>2</sub>O (4 x 7 mL), then treated with CH<sub>2</sub>Cl<sub>2</sub> /MeOH/DIPEA (17: 2: 1, v/v/v) (7 mL) for 40 min. Fmoc deprotection was achieved by treatment with piperidine/DMF (1:4, v/v) (3 x 7 mL), for 4 x 3 min. Peptide couplings were performed relative to the nominal loading, using Fmoc-amino acid (2 eq), PyBOP (1.9 eq) and DIPEA (8 eq) in DMF (5 mL) for 90 min, except for the case of coupling to MePhe when Fmoc-Arg(Z)<sub>2</sub>-OH (2 eq), HATU (2 eq) and DIPEA (4 eq) in DMF (5 mL) for 150 min were used. The resin-bound peptide was *N*- $\alpha$ -deprotected and acetylated by treatment with Ac<sub>2</sub>O/CH<sub>2</sub>Cl<sub>2</sub>/DIPEA (5 mL, 1:8:1 v/v/v), for 45 min at room temperature. The Kaiser test was carried out and the beads were brown, therefore the tripeptide was treated again with Ac<sub>2</sub>O/CH<sub>2</sub>Cl<sub>2</sub>/DIPEA (5 mL, 1:8:1 v/v/v), for 160 min. The resin was washed with DMF (4 x 7 mL), CH<sub>2</sub>Cl<sub>2</sub> (4 x 7 mL), MeOH (4 x 7 mL), Et<sub>2</sub>O (4 x 7 mL). Cleavage from the resin and side-chain protection were achieved by treatment with TFA/TIPS/H<sub>2</sub>O (10 mL, 95:2.5:2.5 v/v/v). All of the solvent was evaporated under vacuum and the residue was dissolved in TFA and precipitated with cold Et<sub>2</sub>O. The solid was isolated by centrifugation, to give (**74**) as a white solid (18 mg, 15%); **RP-HPLC** (analytical system, gradient 1) *R*<sub>t</sub> = 8.7 min. [found (ES<sup>+</sup>) 761.3108 [M+H]<sup>+</sup>, C<sub>38</sub>H<sub>45</sub>N<sub>6</sub>O<sub>11</sub> requires 761.3141].

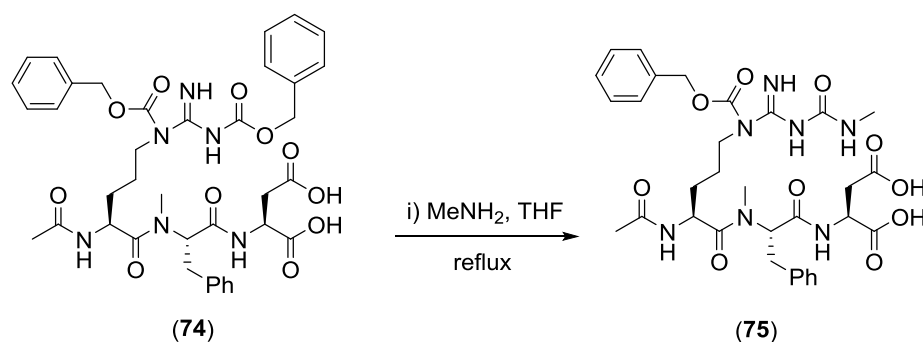
#### Ac-Arg(Z)<sub>2</sub>-MePhe-Asp-OBn (**77**)



The synthesis was carried out manually by Fmoc SPPS. Wang resin (0.354 mg, 0.300 mmol) was pre-swollen with CH<sub>2</sub>Cl<sub>2</sub> (7 mL), for 30 min. Fmoc-Asp-OBn (**76**) (0.522 g, 1.17 mmol) was dissolved in dry CH<sub>2</sub>Cl<sub>2</sub> (4 mL), DMF (3 mL) and DIC (0.75 g, 0.59 mmol) in dry CH<sub>2</sub>Cl<sub>2</sub> (3 mL) was added and then the mixture was stirred for 20 min at 0 °C. All the solvent was

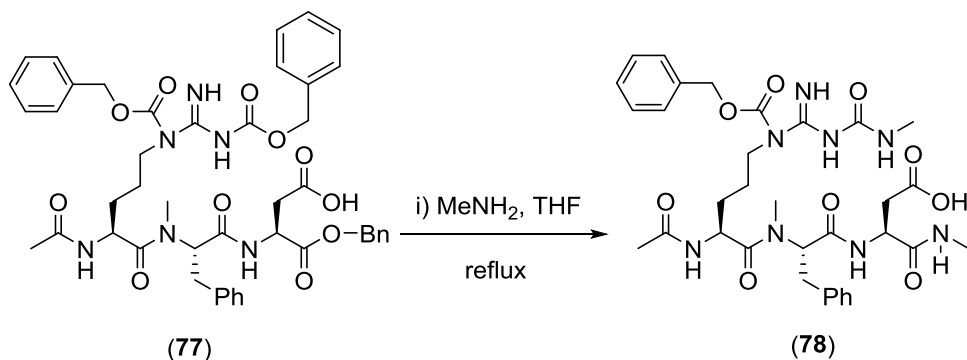
evaporated, and the residue was dissolved in DMF (7 mL). The resin and DMAP (9.5 mg, 78  $\mu\text{mol}$ ) were added to the mixture and stirring was continued at room temperature for 90 min. The resin was washed with DMF (4 x 7 mL),  $\text{CH}_2\text{Cl}_2$  (4 x 7 mL), MeOH (4 x 7 mL),  $\text{Et}_2\text{O}$  (4 x 7 mL). The loading was determined to be 59% based on UV absorption after the Fmoc deprotection. Fmoc deprotection was achieved by treatment with piperidine/DMF (1:4, v/v) (3 x 7 mL), for 4 x 3 min. Peptide couplings were performed using Fmoc-amino acid (2 eq), PyBOP (1.9 eq) and DIPEA (8 eq) in DMF (5 mL) for 90 min, except for the case of coupling to MePhe when Fmoc-Arg(Z)<sub>2</sub>-OH (2 eq), PyBrOP (2 eq) and DIPEA (4 eq) in DMF (5 mL) for 4 x 90 min were used. The resin-bound peptide was *N*- $\alpha$ -deprotected and acetylated by treatment with  $\text{Ac}_2\text{O}/\text{CH}_2\text{Cl}_2/\text{DIPEA}$  (5 mL, 1:8:1 v/v/v), for 1 h at room temperature. The resin was washed with DMF (4 x 7 mL),  $\text{CH}_2\text{Cl}_2$  (4 x 7 mL), MeOH (4 x 7 mL),  $\text{Et}_2\text{O}$  (4 x 7 mL). Cleavage from the resin was achieved by treatment with TFA/TIPS/ $\text{H}_2\text{O}$  (10 mL, 95:2.5:2.5, v/v/v) for 1 h. All of the solvent was removed under vacuum and the tripeptide was dissolved in the minimum quantity of TFA and precipitated with cold  $\text{Et}_2\text{O}$ . The solid was isolated by centrifugation, to give (**77**) as a yellow solid (52 mg, 68%); **RP-HPLC** (analytical system, gradient 1)  $R_t = 10.0$  min. [found (ES<sup>+</sup>) 873.3380  $[\text{M}+\text{Na}]^+$ ,  $\text{C}_{45}\text{H}_{50}\text{N}_6\text{O}_{11}\text{Na}$  requires 873.3429].

#### Attempted preparation of Ac-Arg( Z, CONHMe)-MePhe-Asp-OBn (**75**)



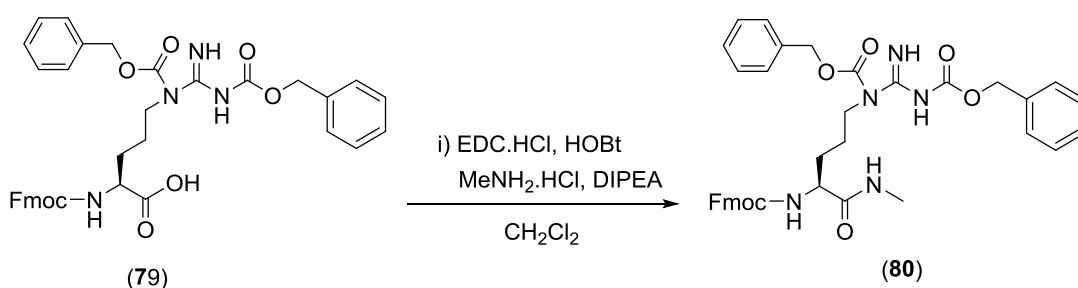
A solution of (**74**) (6.0 mg, 7.8  $\mu\text{mol}$ ) in THF (0.5 mL) was treated with a solution of 2M methylamine in THF (19  $\mu\text{L}$ , 39  $\mu\text{mol}$ ). The reaction mixture was stirred at 65  $^{\circ}\text{C}$  overnight. After 24 h and 48 h respectively, the reaction showed no change as shown by HPLC and extra methylamine (5 eq) was added. After 72 h, HPLC again showed no further change. Mass spectrometry at this stage also showed only the starting material.

### Attempted preparation of Ac-Arg(Z, CONHMe)-MePhe-Asp-CONHMe (**78**)



A solution of (**77**) (21 mg, 24  $\mu\text{mol}$ ) in THF (1 mL) was treated with a 2M solution of methylamine in THF (60  $\mu\text{L}$ , 0.12 mmol). The reaction mixture was stirred at 65°C overnight. After 24 h and 48 h respectively, HPLC showed the partial disappearance of the starting material and formation of a new peak. Extra methylamine (5 eq) was added. After 72 h, little further change was observed. Mass spectrometry did not indicate formation of the desired compound.

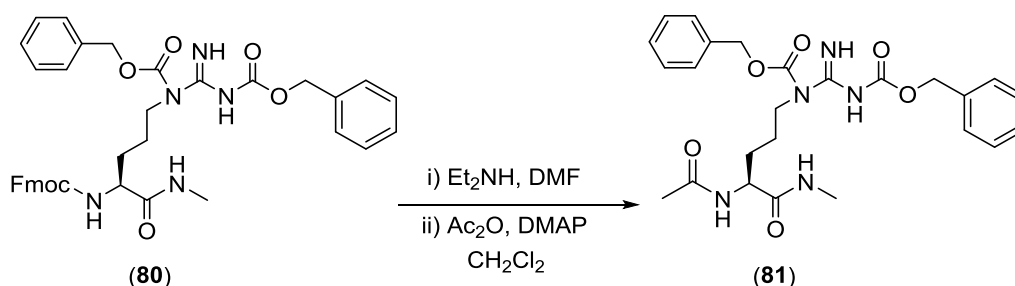
### *N*<sup>δ</sup>-*N*<sup>ε</sup>-Bis(benzyloxycarbonyl)-*N*<sup>α</sup>-(fluorine-9-ylmethoxycarbonyl)-L-arginine-*N*-methlamide (**80**)



Fmoc-Arg(Z)<sub>2</sub>-OH (**79**) (113 mg, 0.170 mmol), EDC.HCl (67 mg, 0.30 mmol), HOBT (87 mg, 0.23 mmol) and MeNH<sub>2</sub>.HCl (116 mg, 0.24 mmol) were dissolved in anhydrous CH<sub>2</sub>Cl<sub>2</sub> (7 mL). The reaction mixture was then treated with DIPEA (0.9 mL, 0.56 mmol) and stirring was continued at room temperature for 16 h. After disappearance of the starting material as shown by TLC (10% MeOH/CH<sub>2</sub>Cl<sub>2</sub>), all of the solvents were evaporated and the crude material was purified by column chromatography using 3-4% MeOH/CH<sub>2</sub>Cl<sub>2</sub> eluent. The desired compound

was isolated (**80**) as a white solid (102 mg, quantitative yield); **Mp** 180-182 °C; **IR**  $\nu_{\text{max}}$  (KBr disc) 3392, 3300 (NH), 2952 (CH), 1721, 1656, 1610 (C=O), 1445 (NH), 1259 (CO); **<sup>1</sup>H NMR** (CDCl<sub>3</sub>, 400 MHz)  $\delta$  1.57-1.78 (4H, m, 2 x H $\beta$ , 2 x H $\gamma$ ), 2.34 (3H, d, *J* 4.6 Hz, NH CH<sub>3</sub>), 3.56-3.63 (1H, m, 1 x H $\delta$ ), 3.89-3.91 (1H, m, 1 x H $\delta$ ), 4.16 (1H, t, *J* 6.3 Hz, Fmoc-CH), 4.19-4.27 (1H, m, Fmoc-CH<sub>2</sub>), 4.35-4.39 (1H, m, Fmoc-CH<sub>2</sub>), 4.53-4.58 (1H, m, H $\alpha$ ), 4.90 (1H, d, *J* 12.3 Hz, CH<sub>2</sub>Ph), 5.05 (1H, d, *J* 12.3 Hz, CH<sub>2</sub>Ph), 5.21-5.28 (2H, m, CH<sub>2</sub>Ph), 6.41 (2H, br, NH), 7.19-7.41 (10H, m, 2 x Ph), 7.44-7.58 (2H, m, Ar-H Fmoc), 7.70-7.75 (2H, m, Ar-H Fmoc), 9.36 (1H, br, NH), 9.48 (1H, br, NH); **<sup>13</sup>C NMR** (CDCl<sub>3</sub>, 101 MHz)  $\delta$  24.35 (CH<sub>2</sub> $\beta$ ), 25.79 (CH<sub>2</sub> $\gamma$ ), 47.22 (CH<sub>2</sub> $\delta$ ), 54.23 (CH<sub>3</sub>N), 66.33 (CH Fmoc), 67.20 (CH $\alpha$ ), 69.01 (CH<sub>2</sub> Fmoc), 119.83, 119.98, 124.77, 127.00, 127.50, 127.62, 128.17, 128.27, 128.32, 128.55, 128.81, 128.93, 128.93 (CH=CH Ar), 134.47, 136.03, 141.31, 143.76, 143.88, 155.68, 156.05, 160.87 (C quaternary), 171.77 (C=O); [found (ES<sup>+</sup>) 700.2777 [M+Na]<sup>+</sup>, C<sub>38</sub>H<sub>39</sub>N<sub>5</sub>O<sub>7</sub>Na requires 700.2747].

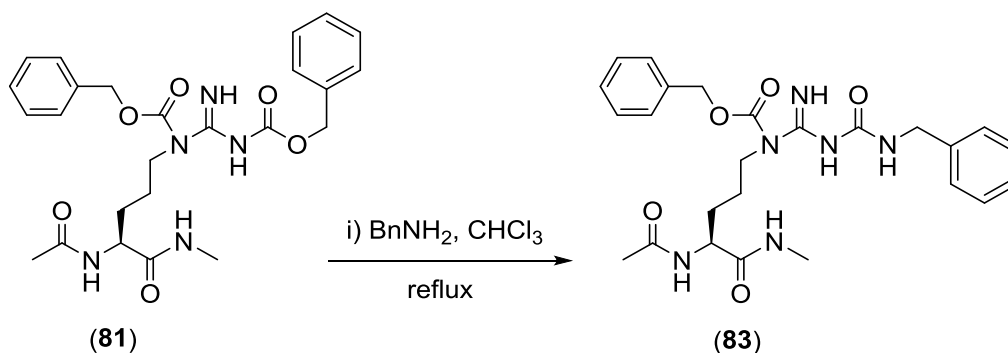
***N* <sup>$\alpha$</sup> -Acetyl-*N* <sup>$\delta$</sup> ,*N* <sup>$\epsilon$</sup> -bis(benzyloxycarbonyl)-L-arginine-*N*-methanamide (**81**)**



Compound (**80**) (73 mg, 0.11 mmol) was dissolved in anhydrous DMF (6 mL) and was treated with Et<sub>2</sub>NH (124  $\mu$ L, 1.10 mmol) at room temperature for 30 min. After consumption of the starting material as shown by TLC, the reaction mixture was then co-evaporated with toluene. The crude material (50 mg, 0.11 mmol) was dissolved in anhydrous CH<sub>2</sub>Cl<sub>2</sub> (5 mL) and Ac<sub>2</sub>O (22.0 mg, 0.22 mmol) and DMAP (13.4 mg, 0.110 mmol) were added and then stirring was continued at room temperature. The reaction was checked by TLC (10% MeOH/CH<sub>2</sub>Cl<sub>2</sub>). After running the reaction for 16 h, the starting material was consumed and all the solvents were evaporated and the crude material purified by column chromatography using 5-10%

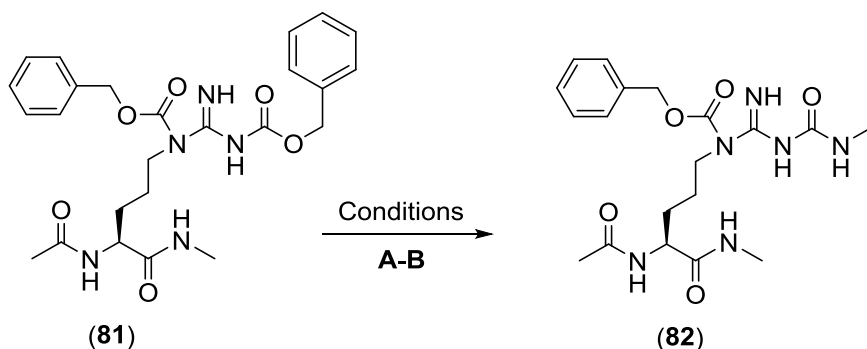
MeOH/CH<sub>2</sub>Cl<sub>2</sub> eluent. The desired compound (**81**) was isolated as a white solid (46 mg, 78%); **Mp** 185-187°C; **IR**  $\nu_{\text{max}}$  (KBr disc) 3403, 3287 (NH), 2949 (CH), 1722, 1641 (C=O), 1254 (CO); **<sup>1</sup>H NMR** (CDCl<sub>3</sub>, 400 MHz)  $\delta$  1.59-1.76 (4H, m, 2 x H $\beta$ , 2 x H $\gamma$ ) 1.91 (3H, s, CH<sub>3</sub>CO), 2.49 (3H, d, *J* 4.6 Hz, CH<sub>3</sub>NH), 3.81-3.88 (1H, m, 1 x H $\delta$ ), 4.05-4.12 (1H, m, 1 x H $\delta$ ), 4.48-4.53 (1H, m, H $\alpha$ ), 5.11 (2H, d, *J* 12.3 CH<sub>2</sub>Ph), 5.18 (2H, *J* 12.3 Hz, CH<sub>2</sub>Ph) 5.24 (2H, s, CH<sub>2</sub>Ph), 6.57-6.58 (1H, br, NHCH<sub>3</sub>), 6.99 (1H, d, *J* 8.2 Hz, NH $\alpha$ ), 7.31-7.39 (10H, m, 2 x Ph), 9.37 (1H, br, NH), 9.49 (1H, br, NH); **<sup>13</sup>C NMR** (CDCl<sub>3</sub>, 101 MHz)  $\delta$  21.27, 22.89, 24.12, 26.48, 42.37, 50.66, 65.49, 67.28, 126.47, 126.52, 126.83, 127.02, 127.09, 132.72, 134.39, 153.96, 159.34, 161.61, 168.16, 170.19; [found (ES<sup>+</sup>) 498.2356 [M+H]<sup>+</sup>, C<sub>25</sub>H<sub>32</sub>N<sub>5</sub>O<sub>6</sub> requires 498.2352].

**Attempted preparation of *N* <sup>$\alpha$</sup> -Acetyl-*N* <sup>$\epsilon$</sup> -(benzylaminocarbonyl)-*N* <sup>$\delta$</sup> -benzyloxycarbonyl-L-arginine-*N*-methylamide (**83**)**



A stirred solution of (**81**) (20 mg, 40  $\mu$ mol) in anhydrous CHCl<sub>3</sub> (2 mL), was treated with benzylamine (8.7  $\mu$ L, 0.08 mmol) and stirring was continued at reflux for 16 h. The solvent was evaporated to give a white solid. The residue was dissolved in CH<sub>2</sub>Cl<sub>2</sub> and purified by column chromatography (MeOH/CH<sub>2</sub>Cl<sub>2</sub> gradient) to give a white solid (18 mg, 92%); **R<sub>f</sub>** 0.4 (10% MeOH/CH<sub>2</sub>Cl<sub>2</sub>). Mass spectrometry showed unchanged starting material.

**Attempted preparation of *N*<sup>α</sup>-Acetyl-*N*<sup>δ</sup>-benzyloxycarbonyl-*N*<sup>ε</sup>-(methylaminocarbonyl)-L-arginine-*N*-methanide (82)**



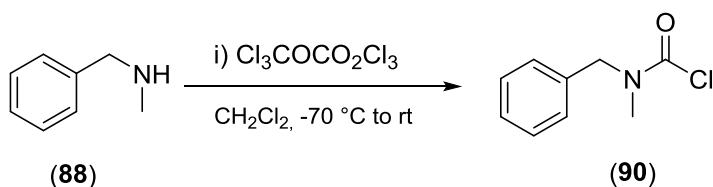
**A:** MeNH<sub>2</sub>, THF, rt, 16 h; **B:** as **A**, using CHCl<sub>3</sub>.

**Method A:** A stirred solution of **(81)** (20 mg, 40 μmol) in anhydrous THF (2 mL), was treated with 2M solution of methylamine in THF (0.1 mL, 0.2 mmol) and stirring was continued at room temperature. The reaction was monitored by TLC. After 4 h the reaction showed no change by TLC and an extra 5 eq of 2M solution of methylamine in THF (0.1 mL, 0.2 mmol) was added. After 16 h, no further change had been indicated by HPLC. Analysis by HPLC and <sup>1</sup>H NMR showed unchanged starting material.

**Method B:** A stirred solution of **(81)** (20 mg, 40 μmol) in anhydrous CHCl<sub>3</sub> (2 mL), was treated with 2M solution of methylamine in THF (0.1 mL, 0.2 mmol) and stirring was continued at room temperature. The reaction was monitored by TLC. After 4 h the reaction showed no change and further 2M methylamine in THF (0.1 mL, 0.2 mmol), was added. After 16 h, again no change was shown by HPLC.

### 7.2.3. Approaches to alternative reagents for the direct conversion of Orn (or simple amines) to Arg(MC) and analogues

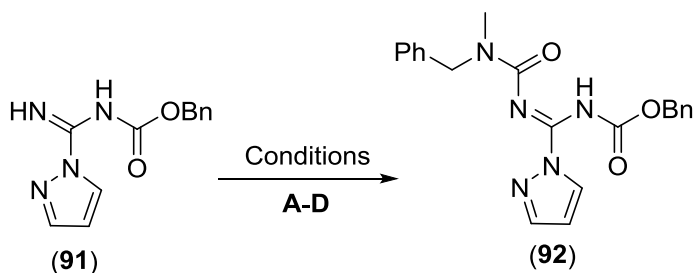
#### *N*-Benzyl-*N*-methylcarbamoyl chloride (**90**)<sup>170</sup>



A solution of bis(trichloromethyl)carbonate (0.78 g, 26 mmol) in anhydrous CH<sub>2</sub>Cl<sub>2</sub> (10 mL) was cooled to -70 °C under N<sub>2</sub> and pyridine (0.62 mL, 7.8 mmol) and *N*-methylbenzylamine (**88**) (1.0 mL, 7.8 mmol) were added. The reaction mixture was allowed to warm up to room temperature and was monitored by TLC (10% MeOH/CH<sub>2</sub>Cl<sub>2</sub>). After running the reaction for 2 h the starting material was consumed, then the reaction was neutralized with 1M aq. HCl (10 mL) and it was extracted with EtOAc (2 x 20 mL). The combined organic extracts were dried over MgSO<sub>4</sub>, filtered and the solvent was evaporated to give an oil that crystallised at room temperature (**90**) (1.56 g, quantitative yield); <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz) δ two rotamers: 2.99 (1.25H, s, minor rotamer CH<sub>3</sub>N), 3.06 (1.75H, s, major rotamer CH<sub>3</sub>N), 4.56 (1.17H, s, major rotamer CH<sub>2</sub>Ph), 4.71 (0.83H, s, minor rotamer CH<sub>2</sub>Ph), 7.25-7.38 (5H, m, Ph).



**Attempted preparation of benzyl -((benzyl(methyl)carbamoyl)imino)(1H-pyrazol-1-yl)methyl)carbamate (92)**



**A:** (**90**), NaH, THF, 0 °C to rt, 3 h; **B:** as **A**, but using fresh NaH; **C:** (**90**), DMAP, DIPEA, CH<sub>2</sub>Cl<sub>2</sub>, rt, 16 h; **D:** (**90**), NaH, THF, 0 °C to rt, 16 h.

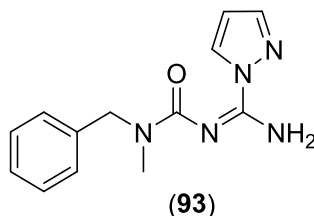
**Method A:** A stirred solution of (**91**) (0.22 g, 0.90 mmol) in anhydrous THF (9 mL) was purged under N<sub>2</sub> and was cooled to 0 °C. 60% NaH (55 mg, 1.4 mmol) was then added. The reaction mixture was stirred at 0 °C for 10 min, then warmed up to room temperature, after which (**90**) (0.50 g, 2.7 mmol) in anhydrous THF (3 mL) was added to the reaction mixture, then the stirring was continued with heating at reflux. The reaction was monitored by TLC (30% EtOAc/petroleum ether). After running the reaction for 3 h, a new spot appeared on TLC. The reaction mixture was diluted into EtOAc (50 mL), then neutralized with sat. aq. NH<sub>4</sub>Cl (30 mL). The organic layer was separated and extracted with H<sub>2</sub>O (50 mL), dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and the solvent evaporated to give a white solid. The crude material was purified by column chromatography using 5-25% EtOAc/ petroleum eluent. Analysis by <sup>1</sup>H NMR indicated the presence of both starting materials.

**Method B:** A stirred solution of (**91**) (110 mg, 0.45 mmol) in anhydrous THF (4.5 mL), was purged under N<sub>2</sub>, cooled to 0 °C, then fresh 60% NaH ( 26 mg, 0.68 mmol) was added. The reaction mixture was stirred at 0 °C for 10 min, then warmed up to room temperature, after which (**90**) (250 mg, 1.36 mmol) in anhydrous THF (3 mL) was added to the reaction mixture *via* a cannula. Stirring was continued overnight. The reaction was monitored by TLC (30% EtOAc/petroleum). After running the reaction for 16 h, a new spot appeared. The reaction mixture was diluted into EtOAc (50 mL), then neutralized with sat. aq. NH<sub>4</sub>Cl (30 mL) and the organic layer was separated and extracted with H<sub>2</sub>O (50 mL), dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and the solvent evaporated to give a white solid. The crude material was purified by column

chromatography using 30-40% EtOAc/ petroleum ether eluent. Analysis by  $^1\text{H}$  NMR revealed both starting materials: (90) and (91).

**Method C:** A stirred solution of (91) (96 mg, 0.39 mmol) in anhydrous  $\text{CH}_2\text{Cl}_2$  (20 mL), was purged under  $\text{N}_2$ , then (90) (180 mg, 0.93 mmol), DMAP (9.6 mg, 78  $\mu\text{mol}$ ) and DIPEA (171  $\mu\text{L}$ , 0.983 mmol), were added to the reaction mixture. Stirring was continued at room temperature overnight, and the reaction was monitored by TLC (30% EtOAc/petroleum ether). After running the reaction for 16 h, a new spot with a lower  $R_f$  appeared. The reaction mixture was neutralized with 1M aq. solution of HCl (30 mL), then extracted with  $\text{CH}_2\text{Cl}_2$  (2 x 20 mL). The combined organic extracts were dried over  $\text{Na}_2\text{SO}_4$ , filtered and the solvents evaporated to give a yellow solid. Analysis by  $^1\text{H}$  NMR was not consistent with formation of the expected product.

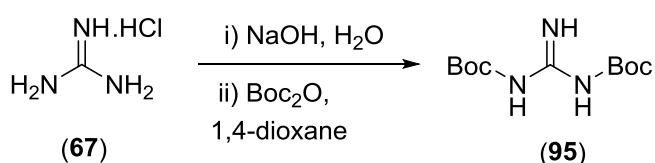
**Method D:** A stirred solution of (91) (220 mg, 0.90 mmol) in freshly distilled THF (9 mL), was purged under  $\text{N}_2$  and cooled to  $0^\circ\text{C}$ . Fresh NaH (131 mg, 3.28 mmol assumed) was added to the reaction mixture, then the stirring was continued at  $0^\circ\text{C}$  for 10 min, followed by warming up to room temperature. Compound (90) (500 mg, 2.73 mmol) in anhydrous THF (3 mL) was added to the reaction mixture *via* a cannula and then stirring was continued overnight. The reaction was monitored by TLC (30% EtOAc/petroleum ether). After running the reaction for 16 h, a new spot with a lower  $R_f$  appeared. The reaction mixture was diluted into EtOAc (50 mL) and then neutralized with sat. aq.  $\text{NH}_4\text{Cl}$  (30 mL). The organic layer was separated and extracted with  $\text{H}_2\text{O}$  (50 mL), dried over  $\text{Na}_2\text{SO}_4$ , filtered and the solvents evaporated to give a brown oil. The crude material was purified by column chromatography using 7-10% EtOAc/petroleum ether eluent to give a yellow oil (193 mg, 84%). Analysis by  $^1\text{H}$  NMR showed the following compound:



***N*-[Benzyl(methyl)carbamoyl]-1*H*-pyrazole-1-carboximidamide (93)**

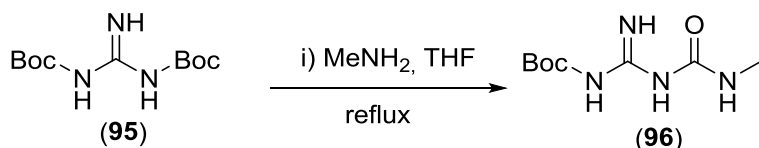
**IR**<sub>vmax</sub> (thin film): 3150, 3128, 3030 (NH), 1650; **<sup>1</sup>H NMR** (CDCl<sub>3</sub>, 400 MHz) δ 3.14 (3H, br, CH<sub>3</sub>N), 4.84 (2H, br, NH<sub>2</sub>), 6.34-6.36 (1H, m, pyrazole ring), 7.29-7.37 (5H, m, Ar), 7.63 (1H, br, pyrazole ring), 8.17-8.19 (1H, m, pyrazole ring); **<sup>13</sup>C NMR** (CDCl<sub>3</sub>, 101 MHz) δ 35.69 (CH<sub>3</sub>N), 53.43 (CH<sub>2</sub> benzylic), 106.42, 125.83, 126.63, 126.85, 127.64, 127.99 (CH Ar), 130.19, 130.85, 135.42, 140.82, 151.33 (C quaternary and pyrazole ring) (C=O); [found (ES<sup>+</sup>) 280.1157 [M+Na]<sup>+</sup>, C<sub>13</sub>H<sub>15</sub>N<sub>5</sub>ONa requires 280.1169].

### 1,3-di-Boc-guanidine (**95**)<sup>164</sup>



A stirred solution of guanidine hydrochloride (**67**) (2.39 g, 25 mmol) in H<sub>2</sub>O (25 mL) was treated with sodium hydroxide (4 g, 0.1 mol) and the reaction mixture was cooled to 0 °C, then 1,4-dioxane (50 mL) was added. Di-*tert*-butyl dicarbonate (12 g, 55 mmol) was added in one portion with vigorous stirring. After 2 h, the reaction was allowed to warm up to room temperature overnight. The solvents were evaporated and then the crude material was purified by column chromatography, using 2% MeOH/CH<sub>2</sub>Cl<sub>2</sub> eluent, to give (**95**) as a white solid (1.4 g, 22%); **Mp** 140-142 °C (lit,<sup>161</sup> 144 °C); **IR** <sub>vmax</sub> (KBr disc) 3415, 3120 (NH), 2983, 2937 (CH), 1732 (C=O), 1681, 1643, 1457, 1401 (NH), 1148 (CO); **<sup>1</sup>H NMR** (CDCl<sub>3</sub>, 400 MHz) δ 1.40 (18H, s, 2 x (C(CH<sub>3</sub>)<sub>3</sub>), 8.33 (3H, br, 3 x NH).

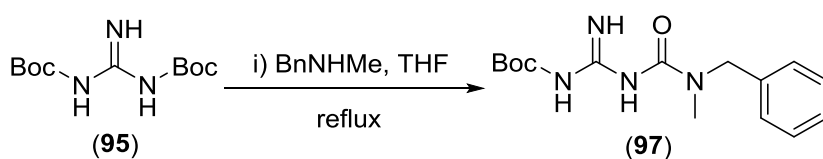
### *tert*-Butoxy(3-methylureido)carbamide (**96**)



A stirred solution of (**95**) (200 mg, 0.760 mmol) in anhydrous THF (1 mL) was purged under an N<sub>2</sub> atmosphere for 10 min, then treated with 2M methylamine in THF (3.8 mL, 7.6 mmol). The reaction mixture was heated at reflux and the reaction was monitored by TLC. After

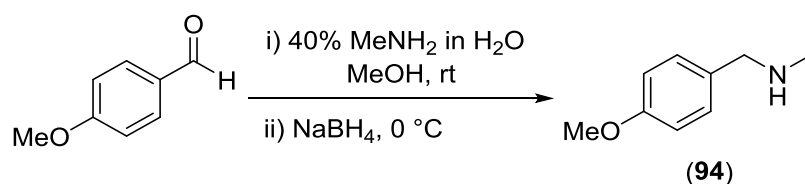
running the reaction for 16 h, a new spot appeared, as shown by TLC (4% MeOH/CH<sub>2</sub>Cl<sub>2</sub>). The solvent were removed under vacuum to give an oil, which was purified by column chromatography, using MeOH/CH<sub>2</sub>Cl<sub>2</sub> eluent. This gave (**96**) (6.7 mg, 4%) plus unchanged starting material (175 mg, 87%). **<sup>1</sup>H NMR** (CDCl<sub>3</sub>, 400 MHz)  $\delta$  1.47 (9H, s, (C(CH<sub>3</sub>)<sub>3</sub>), 2.79 (3H, d, *J* 4.8, NHCH<sub>3</sub>), 5.60 (2H, br, 2 x NH), 8.40 (2H, br, 2 x NH); [found (ES<sup>+</sup>) 224.1006 [M+Na]<sup>+</sup>, C<sub>8</sub>H<sub>16</sub>N<sub>3</sub>NaO<sub>3</sub> requires 224.1006].

***tert*-Butyl (N-benzyl(methyl)carbamoyl]carbamimidoyl)carbamate (**97**)**

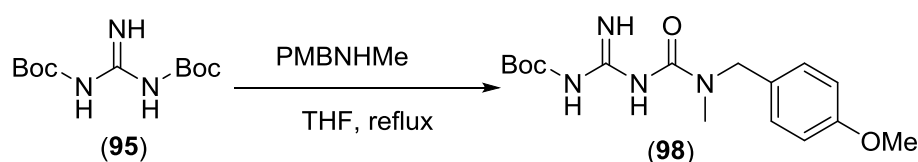


A stirred solution of (**95**) (200 mg, 0.760 mmol) in anhydrous THF (2 mL) was purged under an N<sub>2</sub> atmosphere, then was treated with *N*-benzylmethylamine (**88**) (276 mg, 2.26 mmol) and the reaction mixture was stirred at reflux overnight. The reaction was monitored by TLC. After running the reaction for 72 h, TLC showed that the starting material was replaced by a new lower running spot **R<sub>f</sub>** 0.62 (4% MeOH/CH<sub>2</sub>Cl<sub>2</sub>). The solvent were evaporated and the residue was purified by column chromatography using 0-2% MeOH/CH<sub>2</sub>Cl<sub>2</sub> eluent, to give (**97**) as an oil (182 mg, 78%); **IR**  $\nu_{\text{max}}$  (thin film): 3397, 3251, 3205 (NH), 2977, 2936 (CH), 1719, 1642 (C=O), 1152 (CO); **<sup>1</sup>H NMR** (CDCl<sub>3</sub>, 400 MHz)  $\delta$  two rotamers: 1.47-1.49 (9H, m, (C(CH<sub>3</sub>)<sub>3</sub>), 2.87-3.02 (3H, m, NCH<sub>3</sub>), 4.57 (1H, br, CHPh) 4.71 (1H, br, CHPh), 7.18-7.27 (5H, m, Ph), 7.95 (2H, br, 2 x NH), 9.40 (1H, br, NH); **<sup>13</sup>C NMR** (CDCl<sub>3</sub>, 101 MHz)  $\delta$  two rotamers 27.92 (C(CH<sub>3</sub>)<sub>3</sub>), 28.00 (C(CH<sub>3</sub>)<sub>3</sub>), 32.92 (N-CH<sub>3</sub>), 34.59 (N-CH<sub>3</sub>), 50.99 (CH<sub>2</sub>-Ar), 52.70 (CH<sub>2</sub>-Ar), 82.46 (C(CH<sub>3</sub>)<sub>3</sub>), 82.82 (C(CH<sub>3</sub>)<sub>3</sub>), 126.92, 127.06, 127.46, 128.39 (CH Ar), 138.18, 138.47 (C quaternary), 153.18, 156.57, 164.65 (C=O, C=N); [found (ES<sup>+</sup>) 329.1591 [M+Na]<sup>+</sup>, C<sub>15</sub>H<sub>22</sub>N<sub>4</sub>O<sub>3</sub>Na requires 329.1589].

***tert*-Butyl (*N*-4-methoxybenzyl)methyl)carbamoylcarbamimidoyl)carbamate (**98**)**

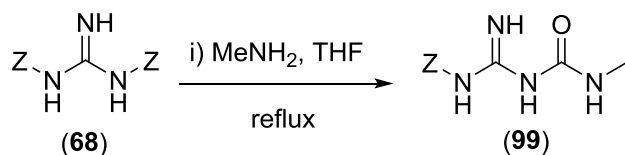


4-Methoxy-*N*-methylbenzylamine (**94**) was prepared according to the procedure of Sunazuka.<sup>147</sup>



A stirred solution of (**95**) (200 mg, 0.76 mmol) in anhydrous THF (2 mL) was purged under an N<sub>2</sub> atmosphere, then was treated with 4-methoxy-*N*-benzylmethylamine (**94**) (345 mg, 2.28 mmol) and the reaction mixture was stirred at reflux overnight. The reaction was monitored by TLC (5% MeOH/CH<sub>2</sub>Cl<sub>2</sub>). After running the reaction for 16 h the starting material disappeared. The solvent was evaporated and the residue was purified by column chromatography using 0-2% MeOH/CH<sub>2</sub>Cl<sub>2</sub> gradient, to give an oil (282 mg). Analysis by <sup>1</sup>H NMR revealed the desired compound plus the presence of the starting material (**94**). The material was again purified using 20-40% EtOAc/petroleum ether eluent to give an oil, which crystallised (**97**) (219 mg, 86%); **Mp** 125-127 °C; **<sup>1</sup>H NMR** (CD<sub>3</sub>OD, 500 MHz) δ two rotamers 1.49 (4.5H, s, (C(CH<sub>3</sub>)<sub>3</sub>), 1.50 (4.5H, s, (C(CH<sub>3</sub>)<sub>3</sub>), 2.78 (1.5H, br, NCH<sub>3</sub> rotamer), 2.94 (1.5H, br, NCH<sub>3</sub> rotamer), 3.31 (1H, br, NH), 3.76 (3H, s, OCH<sub>3</sub>), 4.45 (1H, br, CH<sub>2</sub>Ar rotamer) 4.68 (1H, br, CH<sub>2</sub>Ar rotamer), 6.85 (2H, d, *J* 8.2 Hz, HAr), 7.16 (2H, d, *J* 8.2 Hz, ArH); **<sup>13</sup>C NMR** (CDCl<sub>3</sub>, 101 MHz) δ two rotamers 27.91 (C(CH<sub>3</sub>)<sub>3</sub>), 28.00 (C(CH<sub>3</sub>)<sub>3</sub>), 32.73 (N-CH<sub>3</sub>), 34.40 (N-CH<sub>3</sub>), 50.38 (CH<sub>2</sub>-Ar), 52.08 (CH<sub>2</sub>-Ar), 55.20 (OCH<sub>3</sub>), 82.37 (C(CH<sub>3</sub>)<sub>3</sub>), 82.52 (C(CH<sub>3</sub>)<sub>3</sub>), 113.78, 113.80, 128.39, 128.52, 128.81 130.43 (CH Ar, C quaternary), 156.48, 158.61, 159.01, 164.51 (2 x C=O, 2 x C=N); [found (ES<sup>+</sup>) 359.1688 [M+Na]<sup>+</sup>, C<sub>16</sub>H<sub>24</sub>N<sub>4</sub>NaO<sub>4</sub> requires 359.1690].

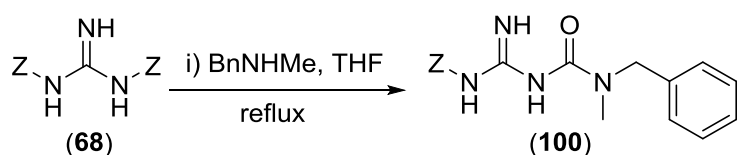
**Attempted preparation of benzyl [*N*-(methylcarbamoyl)carbamimidoyl]carbamate (**99**)**



**Method A:** A stirred solution of **(68)** (1.00 g, 3.05 mmol) in anhydrous THF (20 mL) was purged under an N<sub>2</sub> atmosphere for 10 min, then was treated with 2M solution of methylamine in THF (7.6 mL, 15 mmol) and the reaction mixture was stirred at reflux. The reaction was monitored by TLC (5% MeOH/CH<sub>2</sub>Cl<sub>2</sub>). After running the reaction for 2 h 30 min TLC still showed remaining starting material, therefore another 2.5 eq of methylamine (3.8 mL, 7.6 mmol) was added to the reaction mixture. After running the reaction for 16 h, the solvent was evaporated. Analysis by <sup>1</sup>H NMR revealed the starting material.

**Method B:** A stirred solution of **(68)** (200 mg, 0.611 mmol) in anhydrous THF (1 mL) was purged under an N<sub>2</sub> atmosphere for 10 min, then it was treated with a 2M solution of methylamine in THF (3.05 mL, 6.11 mmol) and the reaction mixture was stirred at reflux overnight. The reaction was monitored by TLC (5% MeOH/CH<sub>2</sub>Cl<sub>2</sub>). After running the reaction for 16 h no change was noticed on TLC. Analysis by <sup>1</sup>H NMR revealed the unchanged starting material.

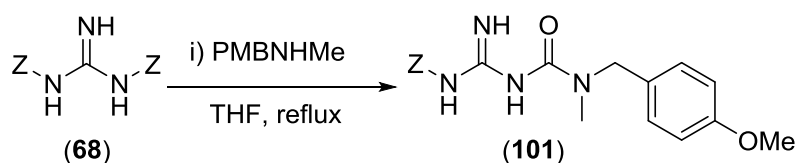
**Benzyl (N-[benzyl(methyl)carbamoyl]cabamimidoyl)carbamate (100)**



A stirred solution of **(68)** (200 mg, 0.611 mmol) in anhydrous THF (4 mL) was purged under an N<sub>2</sub> atmosphere for 10 min, then it was treated with **(88)** (0.24 mL, 6.11 mmol) and the reaction mixture was stirred at reflux. The reaction was monitored by TLC (2% MeOH/CH<sub>2</sub>Cl<sub>2</sub>). After running the reaction for 16 h a new spot appeared on TLC. The solvent was evaporated. The residue was dissolved in CH<sub>2</sub>Cl<sub>2</sub> and purified by column chromatography, using 2-4% MeOH/CH<sub>2</sub>Cl<sub>2</sub> as eluent, to give **(100)** as an oil (18.4 mg, 9%), plus unchanged starting material (144 mg 72%); <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz) δ two rotamers: 2.87 (1.5H, s, NCH<sub>3</sub>), 2.91 (1.5H, s, NCH<sub>3</sub>), 4.50 (2H, br, CH<sub>2</sub>Ph), 5.19 (2H, s, CH<sub>2</sub>Ph), 7.17-7.51 (10H, m,

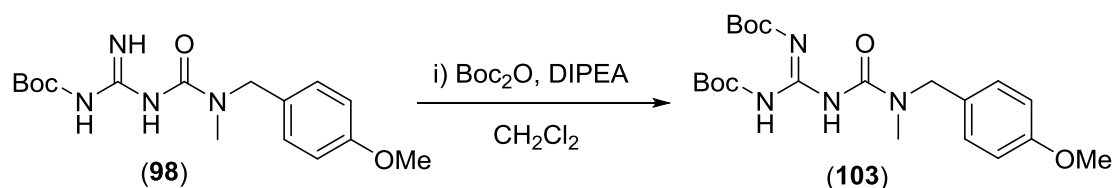
2 x Ph);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 101 MHz)  $\delta$  52.29 (N-CH<sub>3</sub>), 52.57 (N-CH<sub>3</sub>), 67.21 (CH<sub>2</sub>-Ar), 127.26, 127.34, 127.55, 127.81, 127.93, 128.07, 128.15, 128.43, 128.55, 136.72, 137.36 (CH Ar, C quaternary, C=NH), 156.30 (C=O), 156.72 (C=O); found [(ES+) 363.1428 [M+Na]<sup>+</sup>, C<sub>18</sub>H<sub>20</sub>N<sub>4</sub>O<sub>3</sub>Na requires 363.1430].

**Benzyl (N-[(4-methoxybenzyl)methyl]carbamoyl]carbamimidoyl)carbamate (101)**



A stirred solution of **(68)** (500 mg, 1.53 mmol) in anhydrous THF (10 mL) was purged with N<sub>2</sub>, then treated with **(94)** (693 mg, 4.59 mmol) and the stirring continued at reflux overnight. The solvent was evaporated. The residue was dissolved in CH<sub>2</sub>Cl<sub>2</sub> and then purified by column chromatography using petroleum ether to give a white solid (71.7 mg, 13%),  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400 MHz)  $\delta$  two rotamers: 2.85 (1.5H, s, N-CH<sub>3</sub> rotamer), 2.88 (1.5H, s, N-CH<sub>3</sub> rotamer), 3.80 (3H, s, OMe), 4.44 (2H, br, CH<sub>2</sub>-Ar), 5.19 (2H, s, CH<sub>2</sub>-Ar), 6.85-6.87 (2H, m, ArH), 7.10-7.22 (2H, m, ArH), 7.30-7.38 (5H, m, Ph).  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 101 MHz)  $\delta$  two rotamers 33.34 (N-CH<sub>3</sub>), 34.14 (N-CH<sub>3</sub>), 51.68 (CH<sub>2</sub>-Ar), 51.96 (CH<sub>2</sub>-Ar), 55.22 (OCH<sub>3</sub>), 67.14 (CH<sub>2</sub>-Ar), 113.90, 127.80, 127.93, 128.44, 128.70, 129.23, 129.38, 129.49, 136.78, 136.85 (CH Ar, C quaternary), 156.23, 156.64, 158.92 (C=N, C=O); [found (ES+) 393.1533 [M+Na]<sup>+</sup>, C<sub>19</sub>H<sub>22</sub>N<sub>4</sub>O<sub>4</sub>Na requires 393.1535].

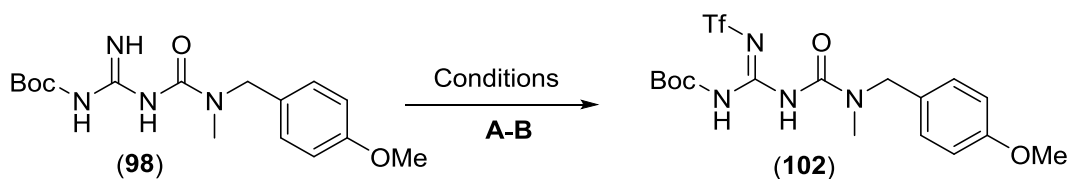
***tert*-butyl(*N*-(*tert*-butoxycarbonyl)-*N'*-[4-methoxybenzyl](methyl)carbamoyl] carbamimidoyl)carbamate (**103**)**



A stirred solution of **(98)** (200 mg, 0.600 mmol) in anhydrous  $\text{CH}_2\text{Cl}_2$  (5 mL) was cooled to 0 °C under an  $\text{N}_2$  atmosphere, then  $\text{Boc}_2\text{O}$  (143 mg, 0.650 mmol) and DIPEA (115  $\mu\text{L}$ , 0.658 mmol), were added to the reaction mixture. The resulting solution was allowed to warm up to room temperature overnight. The reaction was monitored by TLC. After running the reaction for 48 h a new spot with higher  $R_f$  0.80 (1%  $\text{MeOH}/\text{CH}_2\text{Cl}_2$ ) appeared. The solvent was evaporated and the residue was purified by column chromatography using 10-30%  $\text{EtOAc}/\text{petroleum ether}$  eluent. Compound **(103)** was isolated as a white solid (36 mg, 14%), plus the unchanged starting material (132 mg, 66%); **Mp** 120-122 °C;  **$^1\text{H}$  NMR** ( $\text{CDCl}_3$ , 400 MHz)  $\delta$  two rotamers: 1.47 (9H, s,  $(\text{C}(\text{CH}_3)_3)$ ), 1.49 (9H, s,  $(\text{C}(\text{CH}_3)_3)$ ), 2.84 (1.5H, s,  $\text{N}-\text{CH}_3$ ), 3.16 (1.5H, s,  $\text{N}-\text{CH}_3$ ), 3.78 (3H, s, OMe), 4.49 (1H, s,  $\text{Ar}-\text{CH}_2$ ), 4.83 (1H, s,  $\text{Ar}-\text{CH}_2$ ), 6.83 (2H, d,  $J$  8.4 Hz, ArH), 7.19 (1H, d,  $J$  8.5 Hz, ArH), 7.23 (1H, d,  $J$  8.5 Hz, ArH), 10.01-10.21 (1H, m, NH), 12.17-12.47 (1H, m, NH);  **$^{13}\text{C}$  NMR** ( $\text{CDCl}_3$ , 125 MHz)  $\delta$  two rotamers: 27.01 ( $\text{C}(\text{CH}_3)_3$ ), 27.03 ( $\text{C}(\text{CH}_3)_3$ ), 31.62 ( $\text{N}-\text{CH}_3$ ), 33.85 ( $\text{N}-\text{CH}_3$ ), 49.40 ( $\text{Ar}-\text{CH}_2$ ), 51.58 ( $\text{Ar}-\text{CH}_2$ ), 55.23 (OMe), 80.88 ( $\text{C}(\text{CH}_3)_3$ ), 82.57 ( $\text{C}(\text{CH}_3)_3$ ), 112.70, 112.83, 128.02, 128.44, 147.72, 148.19, 148.49, 151.86 (CH Ar, C quaternary), 157.78, 162.30, 162.38 2 x  $\text{C}=\text{O}$ ,  $\text{C}=\text{N}$ ); [found (ES+) 459.2240  $[\text{M}+\text{Na}]^+$ ,  $\text{C}_{21}\text{H}_{32}\text{N}_4\text{O}_6\text{Na}$  requires 459.2219].



**Attempted preparation of *tert*-butyl (*N*-(4-methoxybenzyl)(methyl)carbamoyl-*N'*-(trifluoromethyl)sulfonyl carbamimidoyl)carbamate (102)**



**A:** NaH, chlorobenzene, 0 °C, then Tf<sub>2</sub>O, -45 °C to rt, 16 h; **B:** as **A**, using DIPEA in CH<sub>2</sub>Cl<sub>2</sub>.

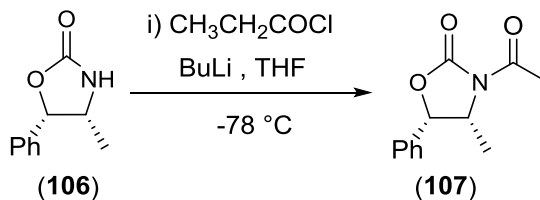
**Method A:** A stirred solution of (98) (69 mg, 0.20 mmol) in anhydrous chlorobenzene (4 mL) was cooled to 0 °C under an N<sub>2</sub> atmosphere, then was treated with 60% NaH (27 mg, 0.66 mmol) and the stirring continued at 0 °C for 1 h, after which the reaction mixture was cooled to -45 °C and triflic anhydride was added and stirring continued for 1 h. The mixture was then allowed to warm to room temperature overnight. TLC showed that the starting material was replaced by a lower running spot **R<sub>f</sub>** 0.50 (4% MeOH/CH<sub>2</sub>Cl<sub>2</sub>). The reaction was quenched with 2M aq. KHSO<sub>4</sub> (7 mL) and extracted with CH<sub>2</sub>Cl<sub>2</sub> (4 x 20 mL). The combined organic extracts were dried over MgSO<sub>4</sub>, filtered and the solvent evaporated to give an oil (47 mg). Analysis by <sup>1</sup>H NMR of the crude product was not consistent with formation of the expected compound.

**Method B:** A stirred solution of (98) (500 mg, 1.49 mmol) in anhydrous CH<sub>2</sub>Cl<sub>2</sub> (10 mL) was cooled to 0 °C under nitrogen atmosphere, then it was treated with DIPEA (0.27 mL, 1.56 mmol) and the stirring continued at 0 °C for 1 h. The reaction mixture was then cooled to -45 °C and triflic anhydride was added and stirring continued for 1 h and then was allowed to warm to room temperature overnight. TLC showed that the starting material was replaced by a lower running spot **R<sub>f</sub>** 0.60 (6% MeOH/CH<sub>2</sub>Cl<sub>2</sub>). The reaction was quenched with 2M aq. KHSO<sub>4</sub> (7 mL) and extracted with CH<sub>2</sub>Cl<sub>2</sub> (4 x 20 mL). The combined organic extracts were dried over MgSO<sub>4</sub>, filtered and the solvent evaporated to give an oil (360 mg). Analysis by <sup>1</sup>H NMR of the crude product was not consistent with formation of the expected compound.

### 7.3. Approaches to Amoa- Key component of banyasin A.

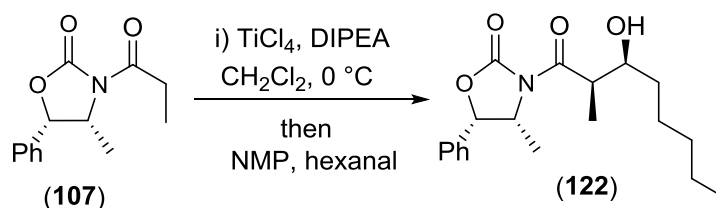
### 7.3.1. Investigation of Ti aldol route towards saturated Amoa

#### (4*R*,5*S*)-4-Methyl-5-phenyl-3-propanoyloxazolidin-2-one (**107**)



A stirred solution of (**106**) (3.00 g, 1.70 mmol) in anhydrous THF (30 mL) was cooled to -78 °C under a N<sub>2</sub> atmosphere, then 2.5 M BuLi in hexanes (7.0 mL, 18.0 mmol) was added dropwise and stirring continued for 30 min. Freshly distilled propanoyl chloride (1.62 mL, 19.0 mmol) was added dropwise to the clear reaction mixture. The resulting solution was left to stir at -78 °C for 30 min and then was allowed to warm to room temperature overnight. TLC showed that the starting material was replaced by a new higher running spot, *R<sub>f</sub>* 0.80 (20% EtOAc/petroleum ether). The reaction was quenched with sat. aq. NH<sub>4</sub>Cl (40 mL), then the organic layer was extracted with aq. NaOH (2 x 20 mL) and brine (2 x 20 mL). The combined organic extracts were dried over MgSO<sub>4</sub>, filtered and the solvent evaporated to give a colourless oil. The crude material was purified by column chromatography (15-30% EtOAc/petroleum ether gradient) to give (**107**) as an oil (3.33 g, 84%); [ $\alpha$ ]<sub>D</sub><sup>20</sup> = +54.1 (c 0.8 in CHCl<sub>3</sub>), (lit [ $\alpha$ ]<sub>D</sub><sup>20</sup> = +44.8 (c 0.25 in CHCl<sub>3</sub>)<sup>252</sup>; IR  $\nu_{\text{max}}$  (thin film): 2983, 2940 (CH), 1781, 1703 (C=O), 1361, 1246 (CO) <sup>1</sup>H NMR (CDCl<sub>3</sub>, 270 MHz)  $\delta$  0.90 (3H, d, *J* 6.6 Hz, CH<sub>3</sub>-oxazolidinone ring), 1.19 (3H, t, *J* 7.4 Hz, CH<sub>3</sub>-CH<sub>2</sub>), 2.90-3.04 (2H, m, CH<sub>3</sub>-CH<sub>2</sub>), 4.76 (1H, *qn*, *J* 6.6 Hz, CH<sub>3</sub>-CH-oxazolidinone ring), 5.65 (1H, d, *J* 7.4 Hz, Ph-CH-oxazolidinone ring), 7.26-7.40 (5H, m, Ph).

**(4*R*,5*S*)-3-[(2*R*,3*S*)-3-Hydroxy-2-methyloctanoyl]-4-methyl-5-phenyl-1,3-oxazolidin-2-one (122)**<sup>184</sup>

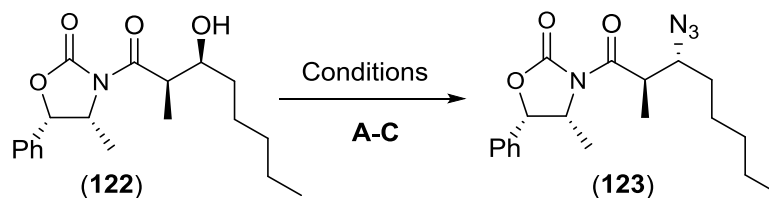


A stirred solution of **(107)** (369 mg, 1.58 mmol) in anhydrous  $\text{CH}_2\text{Cl}_2$  (20 mL), under a  $\text{N}_2$  atmosphere was cooled to  $0^\circ\text{C}$ , 1M  $\text{TiCl}_4$  in  $\text{CH}_2\text{Cl}_2$  solution (1.66mL, 1.66 mmol) was added dropwise and the solution was allowed to stir for 15 min at  $0^\circ\text{C}$ . Freshly distilled DIPEA (303  $\mu\text{L}$ , 1.74 mmol) was added dropwise to the reaction mixture and stirring continued for 40 min. Anhydrous *N*-methylpyrrolidin-2-one (151  $\mu\text{L}$ , 1.58 mmol) was added at  $0^\circ\text{C}$ , followed by the addition of freshly distilled hexanal (379  $\mu\text{L}$ , 3.16 mmol). The deep red colour of the enolate solution disappeared and was replaced by a pale yellow colour. The reaction was monitored by TLC (10% EtOAc/petroleum ether). After 5 h, the starting material was consumed. The reaction was quenched with half-sat. aq.  $\text{NH}_4\text{Cl}$  (40 mL), then the aqueous layer was extracted with  $\text{CH}_2\text{Cl}_2$  (2 x 40 mL). The combined organic extracts were dried over  $\text{Na}_2\text{SO}_4$ , filtered and the solvent evaporated. The crude material was dissolved in  $\text{CH}_2\text{Cl}_2$ , then purified by column chromatography using 15-20% EtOAc/petroleum ether eluent to give **(122)** (388 mg, 74%) as a pale yellow oil, which crystallized at room temperature;  $[\alpha]^{20}_{\text{D}} = +23.4$  (c 0.6 in  $\text{CHCl}_3$ ), (lit  $[\alpha]^{19}_{\text{D}} = +24.9$  (c 0.6 in  $\text{CHCl}_3$ )<sup>184</sup>); **Mp** 82-84  $^\circ\text{C}$ , lit **Mp** 90-91.5  $^\circ\text{C}$ <sup>184</sup>; **IR**  $\nu_{\text{max}}$  (KBr disc): 3452 (OH), 2939, (CH), 1792, 1689 (C=O), 1460 (Ar), 1370, 1343, 1249, 1190 (CO); **<sup>1</sup>H NMR** ( $\text{CDCl}_3$ , 400 MHz)  $\delta$  0.89-0.92 (6H, m,  $\text{CH}_3\text{-CH}_2$ ,  $\text{CH}_3\text{-oxazolidinone ring}$ ), 1.24 (3H, d,  $J$  7.0 Hz,  $\text{CH}_3\text{-CH-CHOH}$ ), 1.36-1.58 (8H, m,  $(\text{CH}_2)_4$ ), 2.83 (1H, br, CH-OH), 3.78 (1H, qd,  $J$  7.0, 2.7 Hz,  $\text{CH}_3\text{-CH-CHOH}$ ), 3.95-3.97 (1H, m,  $\text{CH}_3\text{-CH-CH-OH}$ ), 4.80 (1H, *qn*,  $J$  6.8 Hz,  $\text{CH}_3\text{-CH-oxazolidinone ring}$ ), 5.68 (1H, d,  $J$  7.3 Hz, Ph-CH-oxazolidinone ring), 7.30-7.44 (5H, m, Ph); **<sup>13</sup>C NMR** ( $\text{CDCl}_3$ , 101 MHz)  $\delta$  10.20 ( $\text{CH}_3\text{-CH}_2\text{-CH}_2$ ), 14.02 (CO-CH( $\text{CH}_3$ )-CH), 14.37 ( $\text{CH}_3\text{-oxazolidinone ring}$ ), 22.58 ( $\text{CH}_3\text{-CH}_2\text{-CH}_2$ ), 25.69 ( $\text{CH}_3\text{-CH}_2\text{-CH}_2$ ), 31.78 ( $\text{CH}_2\text{-CH}_2\text{-CHOH}$ ), 33.91 ( $\text{CH}_2\text{-CH}_2\text{-CHOH}$ ), 42.21 (CO-CH-( $\text{CH}_3$ )-CHOH), 54.78 (CO-CH-( $\text{CH}_3$ )-CHOH), 71.62 (CH from oxazolidinone), 78.96 (CH from oxazolidinone), 125.64, 128.76, 128.86 (CH Ar), 133.20 (C quaternary), 152.64 (C=O), 177.45 (CO ester); [found (ES+) 356.1856  $[\text{M}+\text{Na}]^+$ ,  $\text{C}_{19}\text{H}_{27}\text{NO}_4\text{Na}$  requires 356.1852].

### 7.3.2. Displacement reactions

Via Mitsunobu reaction

**Attempted preparation of (4*R*,5*S*)-3-(2*R*,3*R*)-3-azido-2-methyloctanoyl)-4-methyl-5-phenyloxazolidin-2-one (123)**



**A:** Ph<sub>3</sub>P, DEAD, DPPA, CH<sub>2</sub>Cl<sub>2</sub>, rt, 16 h; **B:** as **A**, 0 °C to rt, 16 h; **C:** as **A**, using THF, 16 h.

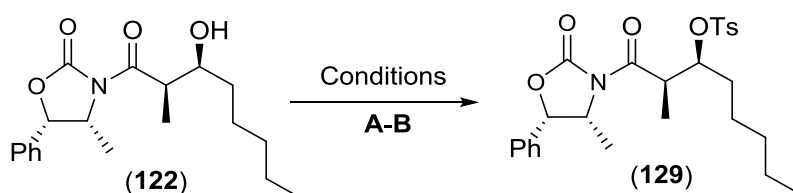
**Method A:** A stirred solution of (**122**) (150 mg, 0.45 mmol), in anhydrous CH<sub>2</sub>Cl<sub>2</sub> (4 mL), was stirred at room temperature. Triphenylphosphine (130 mg, 0.50 mmol) and diethyl azodicarboxylate (78  $\mu$ L, 0.50 mmol) were added and stirring was continued at room temperature for 15 min. The reaction mixture was then treated with diphenylphosphoryl azide (107  $\mu$ L, 0.50 mmol) and stirring was continued at room temperature overnight. After running the reaction for 16 h, a new spot with a higher R<sub>f</sub> appeared, as shown on TLC (15% EtOAc/petroleum). The solvent was evaporated and the crude material was purified by column chromatography, using 0-5% EtOAc/petroleum. This gave (**123**) as a colourless oil (12 mg, 7%) plus unchanged starting material (140 mg, 93%); **IR**  $\nu_{\text{max}}$  (thin film): 2935, 2864 (CH), 2104 (N<sub>3</sub>), 1783, 1699 (CO), 1457, 1345, 1242, 1195 (CO); **<sup>1</sup>H NMR** (CDCl<sub>3</sub>, 400 MHz)  $\delta$  0.78-0.93 (6H, m, CH<sub>3</sub>-CH<sub>2</sub>, CH<sub>3</sub>-oxazolidinone ring), 1.20 (3H, d, *J* 7.0 Hz, CH<sub>3</sub>-CH-CHN<sub>3</sub>), 1.23-1.70 (8H, m, (CH<sub>2</sub>)<sub>4</sub>), 3.69 (1H, td, *J* 9.0, 2.9 Hz, CH-CH(N<sub>3</sub>)-CH<sub>2</sub>), 3.86 (1H, dq, *J* 9.3, 6.9 Hz, CH<sub>3</sub>-CH-CH(N<sub>3</sub>), 4.82 (1H, *qn*, *J* 6.7 Hz, CH<sub>3</sub>-CH-oxazolidinone ring), 5.70 (1H, d, *J* 7.1 Hz, Ph-CH-oxazolidinone ring), 7.30-7.42 (5H, m, Ph); **<sup>13</sup>C NMR** (CDCl<sub>3</sub>, 101 MHz)  $\delta$  12.99 (CH<sub>3</sub>-CH<sub>2</sub>), 13.32 (CH<sub>3</sub>-CO), 13.61 (CH<sub>3</sub> oxazolidinone), 21.50 (CH<sub>3</sub>-CH<sub>2</sub>), 24.24 (CH<sub>2</sub>-CH<sub>2</sub>), 30.24 (CH<sub>2</sub>-CH<sub>2</sub>) 30.56 (CH<sub>2</sub>-(CH)-N<sub>3</sub>), 41.28 (CH(C=O)), 54.01 (CH<sub>2</sub>-(CH)-N<sub>3</sub>), 63.81 (CH oxazolidinone), 78.00 (CH oxazolidinone), 124.58, 127.73 127.78 (CH Ar), 132.13 (C quaternary), 151.59 (C=O), 173.68 (C=O ester); found [(ES<sup>+</sup>) 381.1913 [M+Na]<sup>+</sup>, C<sub>19</sub>H<sub>26</sub>N<sub>4</sub>NaO<sub>3</sub> requires 381.1926].

**Method B:** A stirred solution of (**122**) (140 mg, 0.420 mmol) in anhydrous CH<sub>2</sub>Cl<sub>2</sub> (4 mL), was purged with N<sub>2</sub>, then cooled to 0 °C. Triphenylphosphine (121 mg, 0.46 mmol) and diethyl azodicarboxylate (73 µL, 0.46 mmol) were added and stirring was continued at 0 °C, for 15 min. The reaction mixture was then treated with diphenylphosphoryl azide (100 µL, 0.462 mmol) and allowed to warm up to room temperature overnight. After running the reaction for 16 h, a new spot with a higher R<sub>f</sub> appeared, as shown on TLC (10% EtOAc/petroleum ether). The solvent was evaporated and the crude material was purified by column chromatography, using 0-5% EtOAc/petroleum ether eluent. This gave (**123**) as an oil (9.4 mg, 6%) plus the unchanged starting material (136 mg) alongside with some impurities.

**Method C:** A stirred solution of triphenylphosphine (102 mg, 0.43 mmol) in anhydrous THF (4 mL), was purged with N<sub>2</sub>. Diethyl azodicarboxylate (75 µL, 0.43 mmol) was added and stirring was continued at room temperature for 20 min. The reaction mixture was treated with (**122**) (130 mg, 0.39 mmol) and stirred for 15 min, followed by addition of diphenylphosphoryl azide (93 µL, 0.43 mmol). The reaction was stirred at room temperature overnight. After running the reaction for 16 h, a new spot with a higher R<sub>f</sub> appeared, as showed on TLC (10% EtOAc/petroleum ether). The solvent was evaporated and the crude material was dissolved in CH<sub>2</sub>Cl<sub>2</sub> and purified by column chromatography, using 0-30% EtOAc/petroleum ether eluent. Only starting material was recovered (80 mg, 78%).

*Via the tosylate*

**Attempted preparation of (2*R*,3*S*)-2-methyl-1-((4*R*,5*S*)-4-methyl-2-oxo-5-phenyloxazolidin-3-yl)-1-oxooctan-3-yl 4-methylbenzenesulfonate (**129**)**



**A:** TsCl, pyridine, 0 °C, 3 h; **B:** TsCl, pyridine at 0 °C then heated at 50 °C.

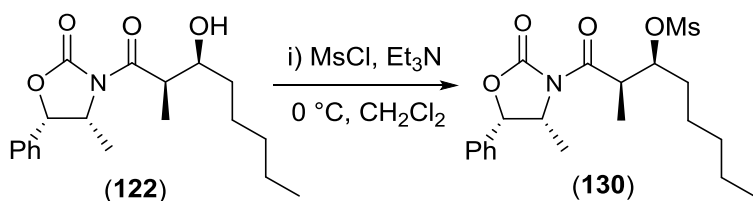
**Method A:** A stirred solution of (**122**) (76 mg, 0.23 mmol) in anhydrous pyridine (4 mL) under a N<sub>2</sub> atmosphere was cooled to 0 °C and treated with tosyl chloride (48 mg, 0.25 mmol). After

running the reaction for 3 h, a new spot with a higher  $R_f$  appeared, as showed on TLC (10% EtOAc/petroleum ether). The reaction was quenched with 1M aq. HCl (20 mL) at 0 °C and was extracted with CHCl<sub>3</sub> (2 x 20 mL). The combined organic layers were washed with brine (2 x 20 mL). The combined organic extracts were dried over MgSO<sub>4</sub>, filtered and the solvent evaporated to give a brown oil (73 mg). Analysis by <sup>1</sup>H NMR indicated the presence of unchanged starting material.

**Method B:** Recovered from **method A (122)** (73 mg, 0.22 mmol) in anhydrous pyridine (2 mL) under a N<sub>2</sub> atmosphere was cooled to 0 °C and treated with a solution of tosyl chloride (46 mg, 0.24 mmol) diluted in anhydrous pyridine (2 mL). Stirring was continued at 0 °C for 5 min and then the mixture was heated at 50 °C, overnight. The reaction was monitored by TLC (10% EtOAc/petroleum ether). After running the reaction for 4 days, a new spot with a higher  $R_f$  appeared. The reaction was quenched with 1M aq. HCl (20 mL) at 0 °C and was extracted with CHCl<sub>3</sub> (3 x 20 mL). The combined organic layers were washed with brine (2 x 20 mL). The combined organic extracts were dried over MgSO<sub>4</sub>, filtered and the solvent evaporated to give a yellow oil (66 mg). Analysis by <sup>1</sup>H NMR indicated the presence of unchanged starting material only.

*Via the mesylate*

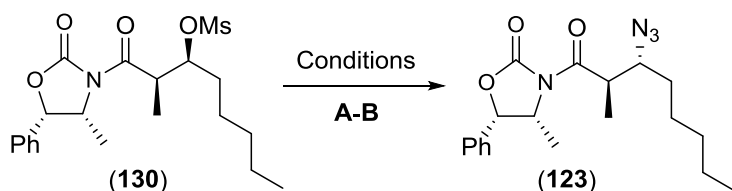
**(2R,3S)-2-Methyl-1-((4R,5S)-4-methyl-2-oxo-5-phenyloxazolidin-3-yl)-1-oxooctan-3-yl methanesulfonate (130)**



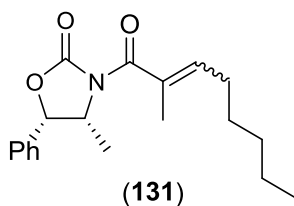
A stirred solution of **(122)** (100 mg, 0.30 mmol) in anhydrous CH<sub>2</sub>Cl<sub>2</sub> (1 mL), was cooled to 0 °C under a N<sub>2</sub> atmosphere then treated with anhydrous Et<sub>3</sub>N (84 µL, 0.60 mmol). After 3 min, methanesulfonyl chloride (24 µL, 0.30 mmol) was added. After running the reaction for 3 h, the reaction mixture was extracted with sat. aq. NaCl (2 x 10 mL) and the aqueous phase was separated, then extracted with CH<sub>2</sub>Cl<sub>2</sub> (2 x 10 mL). The combined organic extracts were dried over MgSO<sub>4</sub>, filtered and the solvent evaporated to give **(130)** (121 mg, 98%) as a brown oil,

which was used for the next step; **IR**<sub>max</sub> (thin film) 1774, 1702 (C=O), 1365, 1174 (SO<sub>2</sub>); **<sup>1</sup>H NMR** (CDCl<sub>3</sub>, 400 MHz) δ 0.80-0.85 (6H, m, CH<sub>3</sub>-CH<sub>2</sub>, CH<sub>3</sub>-oxazolidinone), 1.14 (3H, d, *J* 6.9 Hz, CH<sub>3</sub>-CH-CH(OSO<sub>2</sub>)), 1.24-1.78 (8H, m, (CH<sub>2</sub>)<sub>4</sub>), 2.95 (3H, s, CH<sub>3</sub>-SO<sub>2</sub>), 3.96 (1H, qd, *J* 7.0, 2.8 Hz, CH<sub>3</sub>-CH-CH(OSO<sub>2</sub>)), 4.59-4.66 (1H, m, CH<sub>3</sub>-CH-CH(OSO<sub>2</sub>)), 5.02-5.06 (1H, m, CH<sub>3</sub>-CH-oxazolidinone ring), 5.66 (1H, d, *J* 7.2 Hz, Ph-CH-oxazolidinone ring), 7.21-7.34 (5H, m, Ph).

**Attempted preparation of (4*R*,5*S*)-3-((2*R*,3*R*)-3-azido-2-methyloctanoyl)-4-methyl-5-phenyloxazolidin-2-one (123)**



**Method A:** A stirred solution of **(130)** (120 mg, 0.30 mmol) in anhydrous DMF (2 mL), under N<sub>2</sub>, was treated with sodium azide (57 mg, 0.87 mmol), dissolved in DMSO (1 mL) and 15-crown-5 (6.0 μL, 0.03 mmol). Stirring was continued at 70 °C and the reaction was monitored by TLC (20% EtOAc/petroleum ether). After running the reaction for 3 h, the starting material was consumed. The reaction mixture was allowed to cool to room temperature, then sat. aq. NaCl (5 mL) was added, the mixture was extracted with CH<sub>2</sub>Cl<sub>2</sub> (2 x 10 mL). The combined organic extracts were dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and the solvent evaporated to give a yellow oil. The crude material was purified by column chromatography using 1-5% EtOAc/petroleum ether eluent) to give **(123)** as an oil (28 mg, 27%) and the eliminated product **(131)** as an oil (40 mg, 42%); **IR**<sub>max</sub> (thin film): 2929, 2861 (CH), 2103 (C-C), 1786, 1683 (C=O), 1456 (Ar), 1345, 1197 (CO):



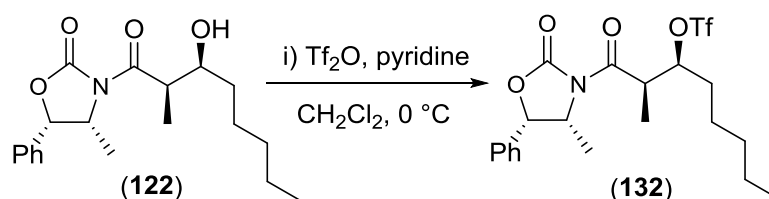
**(4*R*,5*S*)-4-methyl-3-[(2*E*/*Z*)-2-methyl-oct-2-enoyl]-5-phenyl-1,3-oxazolidin-2-one (131)**

**<sup>1</sup>H NMR** (CDCl<sub>3</sub>, 400 MHz)  $\delta$  0.79-0.88 (6H, m, CH<sub>3</sub>-CH<sub>2</sub>, CH<sub>3</sub>-oxazolidinone ring), 1.22-1.29 (4H, m, CH<sub>2</sub>-CH<sub>2</sub>), 1.35-1.41 (2H, m, CH<sub>2</sub>), 1.83 (3H, d, *J* 1.3 Hz, CH<sub>3</sub>-C(C=O)=CH), 2.11 (2H, q, *J* 7.2 Hz C=CH-CH<sub>2</sub>-CH<sub>2</sub>), 4.62-4.68 (1H, m, CH<sub>3</sub>-CH-oxazoline ring), 5.58 (1H, d, *J* 7.4 Hz, Ph-CH-oxazolidinone ring), 5.97-6.01 (1H, m, CH<sub>3</sub>-C(C=O)=CH-CH<sub>2</sub>), 7.20-7.23 (2H, m, H-Ar), 7.29-7.36 (2H, m, H-Ar); [found (ES<sup>+</sup>) 316.1892 [M+H]<sup>+</sup>, C<sub>19</sub>H<sub>26</sub>NO<sub>3</sub> requires 316.1907].

**Method B:** A stirred solution of (**130**) (66 mg, 0.16 mmol) in anhydrous DMF (3 mL), under N<sub>2</sub>, was treated with sodium azide (31 mg, 0.87 mmol), followed by 15-crown-5 (334  $\mu$ L, 0.016 mmol). Stirring was continued at 40 °C and the reaction was monitored by TLC (20% EtOAc/petroleum ether). After running the reaction for 15 min, a TLC showed a new spot with higher R<sub>f</sub>, plus the starting material. The reaction mixture was allowed to cool to room temperature, then sat. aq. NaCl (5 mL) was added and the mixture was extracted with CH<sub>2</sub>Cl<sub>2</sub> (2 x 10 mL). The combined organic extracts were dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and the solvent evaporated to give a yellow oil. The crude material was purified by column chromatography using 1-7% EtOAc/petroleum eluent to give eliminated product (**131**) as an oil (9 mg, 18%) and also unchanged (**127**) (26 mg, 39%).

#### *Displacement of the triflate*

**(2*R*,3*S*)-2-methyl-1-((4*R*,5*S*)-4-methyl-2-oxo-5-phenyloxazolidin-3-yl)-1-oxooctan-3-yl trifluoromethanesulfonate (**132**)**

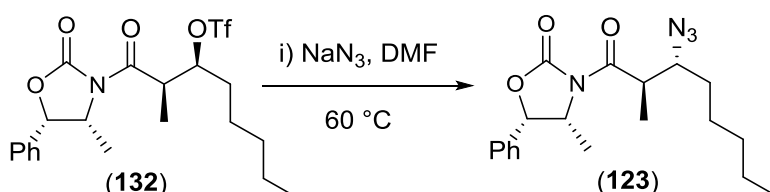


A stirred solution of (**122**) (50 mg, 0.15 mmol) in anhydrous CH<sub>2</sub>Cl<sub>2</sub> (2 mL) under N<sub>2</sub>, was cooled to 0 °C and then was treated with anhydrous pyridine (14  $\mu$ L, 0.18 mmol). Triflic anhydride (28  $\mu$ L, 0.16 mmol) was added to the reaction mixture and the stirring was continued at 0 °C. The reaction was monitored by TLC (10% EtOAc/petroleum ether). After running the reaction mixture for 2 h 30 min, the starting material was consumed as shown by TLC. The reaction was quenched with cold 1M aq. HCl (10 mL) and sat. aq. NaHCO<sub>3</sub> (10 mL), then the



aqueous layer was extracted with CH<sub>2</sub>Cl<sub>2</sub> (2 x 20 mL). The combined organic extracts were dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and the solvent was evaporated to give (**132**) (43 mg, 62%) as an oil, which was used immediately.

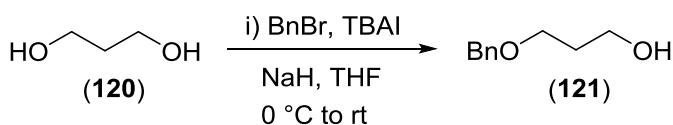
**Attempted preparation of (4*R*,5*S*)-3-((2*R*,3*R*)-3-azido-2-methyloctanoyl)-4-methyl-5-phenyloxazolidin-2-one (**123**)**



A stirred solution of (**132**) (43 mg, 0.09 mmol) in anhydrous DMF (5 mL), was treated with sodium azide (120 mg, 1.84 mmol) and stirring was continued at 60 °C. The reaction was monitored by TLC (15% EtOAc/petroleum ether). After running the reaction for 16 h, the starting material was consumed as shown by TLC. The reaction mixture was diluted in H<sub>2</sub>O (10 mL), then extracted with CH<sub>2</sub>Cl<sub>2</sub> (3 x 20 mL). The combined organic extracts were dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and the solvent evaporated to give a brown oil. The crude material was dissolved in CH<sub>2</sub>Cl<sub>2</sub>, then purified by column chromatography (EtOAc/petroleum ether gradient). Analysis by <sup>1</sup>H NMR revealed mostly eliminated products.

### 7.3.3. Aldol route towards unsaturated Amoa

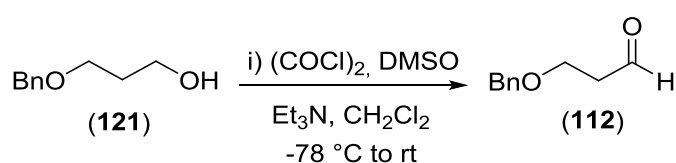
#### 3-(Benzyloxy)propanol<sup>253</sup> (**121**)



A stirred solution of propane-1,3-diol (**120**) (15.0 mL, 214 mmol) in anhydrous THF (130 mL), was cooled to 0 °C and benzyl bromide (8.50 mL, 71.5 mmol), tetra-*n*-butylammonium iodide (5.28 g, 14.0 mmol) and NaH 60% dispersion in mineral oil (3.0 g, 75 mmol) were added. The reaction mixture was allowed to warm to room temperature overnight. The reaction mixture

was washed with sat. aq.  $\text{NH}_4\text{Cl}$  (100 mL) and extracted with EtOAc/Et<sub>2</sub>O (1:1) (4 x 100 mL). The organic extracts were dried over  $\text{MgSO}_4$ , filtered and the solvents evaporated. The crude material was purified by column chromatography using 35-60% EtOAc/petroleum ether eluent to give (**121**) as an oil (9.1 g, 76%); <sup>1</sup>H NMR ( $\text{CDCl}_3$ , 400 MHz)  $\delta$  1.87 (2H, *qn*, *J* 5.8 Hz, O-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>OH), 2.31 (1H, br, OH), 3.66 (2H, t, *J* 5.9 Hz, O-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>OH), 3.78 (2H, t, *J* 5.8 Hz, O-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>OH), 4.52 (2H, s, O-CH<sub>2</sub>-Ph), 7.26-7.37 (5H, m, Ph).

### 3-(Benzyloxy)propanal (**112**)<sup>179, 180</sup>

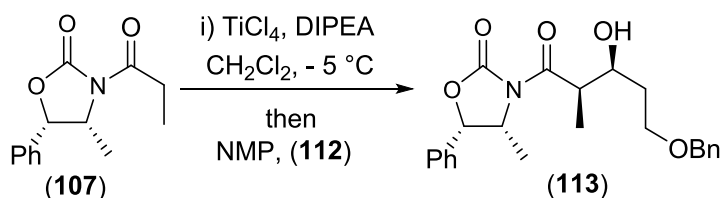


**Method A**<sup>179</sup>: A stirred solution of oxalyl chloride (3.28 mL, 6.56 mmol) in anhydrous  $\text{CH}_2\text{Cl}_2$  (20 mL) was cooled to  $-50^\circ\text{C}$ , DMSO (0.94 mL, 13 mmol) was added dropwise, and stirring continued for 5 min. A solution of 3-(benzyloxy) propanol (**121**) (1.00 g, 6.02 mmol) in anhydrous  $\text{CH}_2\text{Cl}_2$  (5 mL) was added to the reaction mixture *via* a cannula. After 15 min  $\text{Et}_3\text{N}$  (4.17 mL, 30.1 mmol) was added dropwise to the reaction mixture and the stirring continued at  $-50^\circ\text{C}$  for 10 min, then the reaction was allowed to warm up to room temperature. The reaction was monitored by TLC (15% EtOAc/petroleum ether). After running the reaction for 18 h, a new spot with the higher  $R_f$  appeared on TLC. The reaction was quenched with sat. aq.  $\text{NH}_4\text{Cl}$  (10 mL), then the aqueous layer was extracted with  $\text{CH}_2\text{Cl}_2$  (2 x 20 mL). The combined organic layers were washed with sat aq.  $\text{NaHCO}_3$  (2 x 10 mL) and brine (10 mL). The combined organic layers were dried over  $\text{Na}_2\text{SO}_4$ , filtered and the solvent evaporated to give a yellow oil. The crude material was dissolved in  $\text{CH}_2\text{Cl}_2$ , then purified by column chromatography, using EtOAc/petroleum ether eluent, to give (**112**) (236 mg, 24%), plus unchanged starting material.

**Method B**<sup>180</sup>: A stirred solution of oxalyl chloride (3.3 mL, 6.6 mmol) in anhydrous  $\text{CH}_2\text{Cl}_2$  (60 mL) was cooled to  $-78^\circ\text{C}$ , DMSO (0.94 mL, 13.2 mmol) was added then stirring continued for 10 min. (**121**) (1.00 g, 6.02 mmol) was added *via* cannula and stirring continued for 15 min.  $\text{Et}_3\text{N}$  (4.2 mL, 30.0 mmol) was added and stirring continued at  $-78^\circ\text{C}$ , for 5 min, then the reaction mixture was allowed to warm up to room temperature. The reaction was monitored by TLC (15% EtOAc/petroleum ether). After running the reaction for 16 h, a new spot with higher

$R_f$  appeared on TLC. The reaction was quenched with sat. aq.  $\text{NH}_4\text{Cl}$  (10 mL), then the aqueous layer was extracted with  $\text{CH}_2\text{Cl}_2$  (2 x 20 mL). The combined organic layers were washed with sat. aq.  $\text{NaHCO}_3$  (2 x 10 mL) and brine (10 mL). The combined organic layers were dried over  $\text{Na}_2\text{SO}_4$ , filtered and the solvent evaporated to give a yellow oil. The crude material was dissolved in  $\text{CH}_2\text{Cl}_2$ , then purified by column chromatography, using EtOAc/petroleum ether eluent, to give **(112)** (456 mg, 46%), plus unchanged starting material (283 mg, 28%);  $^1\text{H NMR}$  ( $\text{CDCl}_3$ , 400 MHz)  $\delta$  2.68 (2H, td,  $J$  6.1, 4.0 Hz, O- $\text{CH}_2$ - $\text{CH}_2$ -CHO), 3.81 (2H, t,  $J$  6.1 Hz, O- $\text{CH}_2$ - $\text{CH}_2$ -CHO), 4.52 (2H, s, O- $\text{CH}_2$ -Ph), 7.26-7.37 (5H, m, Ph), 9.79 (1H, t,  $J$  4.0 Hz, CHO).

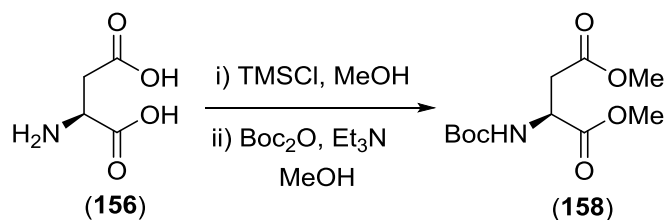
**(4*R*,5*S*)-3-[(2*R*,3*S*)-5-(Benzyloxy)-3-hydroxy-2-methylpentanoyl]-4-methyl-5-phenyl-1,3-oxazolidin-2-one (113)**



A stirred solution of **(107)** (164 mg, 0.70 mmol) in anhydrous  $\text{CH}_2\text{Cl}_2$  (2 mL), under a  $\text{N}_2$  atmosphere was cooled to  $-5\text{ }^\circ\text{C}$ . 1M  $\text{TiCl}_4$  in  $\text{CH}_2\text{Cl}_2$  solution (0.730 mL, 0.735 mmol) was added dropwise and the solution was allowed to stir for 15 min at  $0\text{ }^\circ\text{C}$ . Freshly distilled DIPEA (134  $\mu\text{L}$ , 0.77 mmol) was added dropwise to the reaction mixture and stirring continued for 40 min. Anhydrous *N*-methylpyrrolidin-2-one (67  $\mu\text{L}$ , 0.70 mmol) was added at  $-5\text{ }^\circ\text{C}$ , followed by the addition of freshly made **(112)** (230 mg, 1.40 mmol). The deep red colour of the enolate solution disappeared and was replaced by a pale yellow colour. The reaction was monitored by TLC (10% EtOAc/petroleum ether). After 4 h of reaction the starting material was consumed. The reaction was quenched with half-sat. aq.  $\text{NH}_4\text{Cl}$  (2 x 20 mL), then the aqueous layer was extracted with  $\text{CH}_2\text{Cl}_2$  (2 x 40 mL). The combined organic layers were dried over  $\text{Na}_2\text{SO}_4$ , filtered and the solvent evaporated to give a pale yellow solid. The crude material was dissolved in  $\text{CH}_2\text{Cl}_2$ , then purified by column chromatography using EtOAc/petroleum ether eluent to give **(113)** (108 mg, 39%), as an oil, and a mixture of both starting materials (144 mg);  $[\alpha]^{29\text{D}} = +5.9$  (c 1 in  $\text{CHCl}_3$ ), (lit  $[\alpha]^{29\text{D}} = +8.6$  (c 1.2 in  $\text{CHCl}_3$ )<sup>181</sup> IR  $\nu_{\text{max}}$  (thin film): 3498 (OH), 2943, 2864 (CH), 2319, 2253 (C=C), 1777, 1694 (C=O), 1357 (CO);  $^1\text{H NMR}$  ( $\text{CDCl}_3$ , 400 MHz)  $\delta$  0.88 (3H, d,  $J$  6.6 Hz,  $\text{CH}_3$ -oxazoline ring), 1.26 (3H, d,  $J$  7.0 Hz,  $\text{CH}_3$ -CH), 1.74-1.92

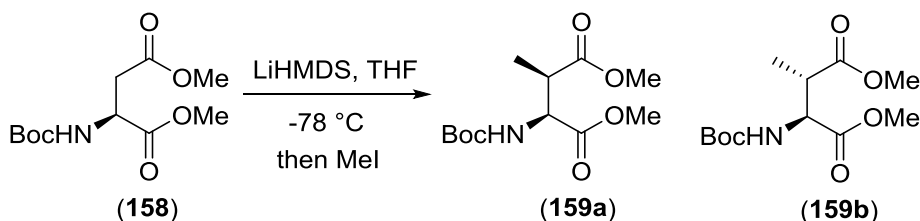
(2H, m, -CHOH-CH<sub>2</sub>-CH<sub>2</sub>-O-), 3.28 (1H, br, CH-OH), 3.64-3.75 (2H, m, CH<sub>2</sub>-CH<sub>2</sub>-O-CH<sub>2</sub>-Ar), 3.85 (1H, dq, *J* 7.0, 3.8 Hz, CH<sub>3</sub>-CH-CHOH), 4.17-4.19 (1H, m, CH-CH(OH)-CH<sub>2</sub>), 4.52 (2H, s, CH<sub>2</sub>-Ph), 4.76 (1H, *qn*, *J* 7.0 Hz, CH<sub>3</sub>-CH-oxazolidinone ring), 5.63 (1H, d, *J* 7.2 Hz, Ph-CH-oxazolidinone ring), 7.26-7.43 (10H, m, 2 x Ph); <sup>13</sup>C NMR (CDCl<sub>3</sub>, 101 MHz) δ 10.96 (CH<sub>3</sub>-oxazolidinone ring), 14.34 (CH<sub>3</sub>-CH), 30.90 (CH<sub>2</sub>-CH<sub>2</sub>-O), 33.73 (CH<sub>2</sub>-CH<sub>2</sub>-O), 54.81 (PhCH<sub>2</sub>-O-), 68.32 (CH-CO), 70.46 (-CHOH-), 73.21 (CH<sub>3</sub>-CH-oxazolidinone ring), 78.84 (Ph-CH-oxazolidinone ring), 125.58, 127.65, 127.82, 128.39, 128.69, 128.75, (CH Ph), 133.16, 138.03 (C quaternary), 152.66 (C=O), 176.38 (C=O ester); [found (ES<sup>+</sup>) 398.1959 [M+H]<sup>+</sup>, C<sub>23</sub>H<sub>28</sub>N<sub>1</sub>O<sub>5</sub> requires 398.1967].

## 7.4. Chiral pool route towards synthesis of Amoa

**Boc-Asp(OMe)-OMe (158)**<sup>254</sup>

A stirred anhydrous of MeOH (15 mL), was cooled to 0 °C and TMSCl (47.6 mL, 376 mmol) was added, then L-aspartic acid (**156**) (10 g, 75 mmol) was added in small fractions to the reaction mixture and the stirring continued at room temperature. After 48 h reaction, the solvent was evaporated, to give the crude diester. A solution of diester (75 mmol) in anhydrous MeOH (200 mL), was cooled to 0 °C and the reaction mixture was treated with Et<sub>3</sub>N (68 mL, 488 mmol) and Boc<sub>2</sub>O (18 g, 83 mmol). The reaction was allowed to warm up to room temperature, overnight. After running the reaction for 16 h, TLC showed disappearance of the starting material and the reaction mixture was diluted in EtOAc (150 mL) and H<sub>2</sub>O (150 mL). The aqueous phase was separated and extracted with CH<sub>2</sub>Cl<sub>2</sub> (100 mL). The combined organic layers were then extracted with H<sub>2</sub>O (3 x 100 mL), dried over MgSO<sub>4</sub>, filtered and the solvent was evaporated to give a pale yellow solid. The crude material was dissolved in CH<sub>2</sub>Cl<sub>2</sub> and then was purified by column chromatography using 2% MeOH/CH<sub>2</sub>Cl<sub>2</sub> eluent to give (**158**) as a pale yellow solid (10.4 g, 53%); **IR**  $\nu_{\text{max}}$  (KBr disc): 3407, (NH), 2981(CH), 1741, 1705 (C=O), 1349, 1163 (CO); **Mp** 58-60 °C, (lit<sup>255</sup> 56 °C); **<sup>1</sup>H NMR** (CDCl<sub>3</sub>, 400 MHz)  $\delta$  1.43 (9H, s, (C(CH<sub>3</sub>)<sub>3</sub>), 2.80 (1H, dd, *J* 16.9, 4.7 Hz, 1H $\beta$ ), 2.95-3.00 (1H, m, 1H $\beta$ ), 3.67 (3H, s, CO<sub>2</sub>Me), 3.74 (3H, s, CO<sub>2</sub>Me), 4.55-4.56 (1H, m, H $\alpha$ ), 5.46-5.47 (1H, m, NH).

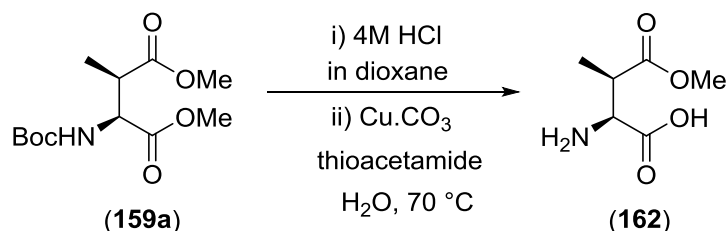
(2*S*,3*R*) and (2*S*,3*S*)-dimethyl-2-((tert-butoxycarbonyl)amino)-3-methylsuccinate<sup>198</sup>  
(**159a**) and (**159b**)



A stirred solution of **(158)** (1.00 g, 3.82 mmol) in anhydrous THF (10 mL) was cooled to -78 °C under a N<sub>2</sub> atmosphere, then 1M LiHMDS in THF (7.64 mL, 7.64 mmol) was added to the reaction mixture and stirring continued for 2 h. Iodomethane (4.58 mmol, 0.28 mL) was added to the reaction mixture. The resulting solution was left to stir at -78 °C for 6 h and then was allowed to warm up at room temperature overnight. TLC showed that the starting material was replaced by two new higher running spots, **R<sub>f</sub>** 0.60 and **R<sub>f</sub>** 0.70 (30% EtOAc/petroleum ether). The reaction was quenched with 3M aq. HCl (10 mL) to pH 2-3 and then the organic layer was washed with 2M aq. Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> (10 mL). The combined organic extracts were dried over MgSO<sub>4</sub>, filtered and the solvent evaporated to give a brown coloured oil. The crude material was dissolved in CH<sub>2</sub>Cl<sub>2</sub> and then purified by column chromatography using 0-12% EtOAc/petroleum ether eluent to give **(159a)** as a yellow oil (183 mg, 17%); **R<sub>f</sub>** 0.60 TLC (30% EtOAc/petroleum ether); <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz) δ 1.24 (3H, d, *J* 7.2 Hz, CH(CH<sub>3</sub>)CO<sub>2</sub>Me) 1.43 (9H, s, (C(CH<sub>3</sub>)<sub>3</sub>), 3.21-3.24 (1H, m, 1H<sub>β</sub>), 3.66 (3H, s, CO<sub>2</sub>Me), 3.72 (3H, s, CO<sub>2</sub>Me), 4.49-4.53 (1H, m, 1H<sub>α</sub>), 5.39-5.42 (1H, m, NH); <sup>13</sup>C NMR (CDCl<sub>3</sub>, 101 MHz) δ 13.46 (CH(CH<sub>3</sub>)CO<sub>2</sub>Me), 28.20 ((C(CH<sub>3</sub>)<sub>3</sub>), 41.38 (CH(CH<sub>3</sub>)CO<sub>2</sub>Me), 51.96 (C O<sub>2</sub>Me), 52.45 (CO<sub>2</sub>Me), 55.30 (CHNHBoc), 79.98 (C(CH<sub>3</sub>)<sub>3</sub>), 155.90 (C=O), 171.47 (C=O), 174.16 (C=O); [found (ES<sup>+</sup>) 298.1253 [M+Na]<sup>+</sup>, C<sub>12</sub>H<sub>21</sub>NO<sub>6</sub>Na requires 298.1261].

Minor isomer as an oil **(159b)** **R<sub>f</sub>** 0.7 TLC (30% EtOAc/petroleum ether) (13 mg, 7%); <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz) δ 1.20 (3H, d, *J* 7.3 Hz, CH(CH<sub>3</sub>)CO<sub>2</sub>Me) 1.43 (9H, s, (C(CH<sub>3</sub>)<sub>3</sub>), 2.95-2.98 (1H, m, 1H<sub>β</sub>), 3.69 (3H, s, CO<sub>2</sub>Me), 3.74 (3H, s, C O<sub>2</sub>Me), 4.63-4.64 (1H, m, H<sub>α</sub>), 5.23-5.25 (1H, m, NH).

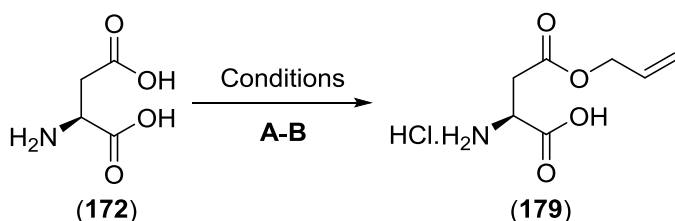
#### Attempted preparation of (2*S*,3*R*)-2-amino-4-methoxy-3-methyl-4-oxobutanoic acid<sup>200</sup> (**162**)



Compound **(159a)** (160 mg, 0.58 mmol) was treated with 4M HCl in dioxane (1 mL) and stirred at room temperature for 30 min. The reaction was checked by TLC (15% EtOAc/petroleum ether), which showed that the starting material disappeared, to give the amine hydrochloride

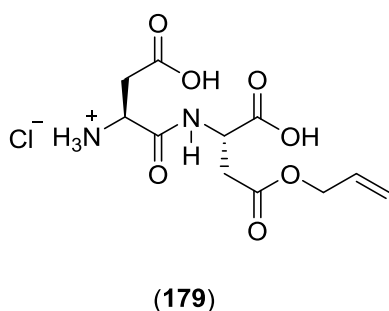
salt. The solvents were evaporated. A stirred solution of the crude salt in H<sub>2</sub>O (10 mL) was treated with basic copper carbonate (154 mg, 0.70 mmol) and stirred at 70 °C for 3 h. The reaction mixture was then filtered through Celite and washed with H<sub>2</sub>O (20 mL). Thioacetamide (79 mg, 1.05 mmol) was added to the reaction mixture and the stirring continued at room temperature overnight. After running the reaction for 16 h, the reaction mixture was quenched with 3M aq. HCl (4 mL) and then the resulting mixture was filtered through Celite. The filtrate was then basified with 3M aq. NaOH (7 mL) and then the precipitate formed was removed by filtration. The solvents were evaporated to give a white solid. Analysis by <sup>1</sup>H NMR was not consistent with formation of the expected product.

**(2S)-4-(Allyloxy)-2-amino-4-oxobutanoic acid (180)**<sup>203, 204</sup>



**A:** Allyl alcohol, TMSCl, 0 °C to rt, 18 h; **B:** as **A**, using acetyl chloride, 18 h.

**Method A**<sup>203</sup>: A stirred solution of L-aspartic acid (**172**) (5.3 g, 40 mmol) in distilled allyl alcohol (30 mL, 441 mmol) was cooled to 0 °C and TMSCl (15.2 mL, 120 mmol) was added. The resulting mixture was allowed to warm to room temperature overnight. After running the reaction for 18 h, the reaction mixture was cooled to 0 °C and then cold diethyl ether (200 mL) was added to the reaction mixture. The white precipitate formed was collected by filtration and washed with cold diethyl ether (2 x 20 mL) to give a white solid (6.7 g, quantitative yield); **Mp** above 230 °C; **IR**  $\nu_{\text{max}}$  (KBr disc) 3397 (NH), 3088, 2987 (OH), 1730, 1710, (C=O), 1567, 1505 (NH), 1279 (CO); Analysis by <sup>1</sup>H NMR showed the following compound:

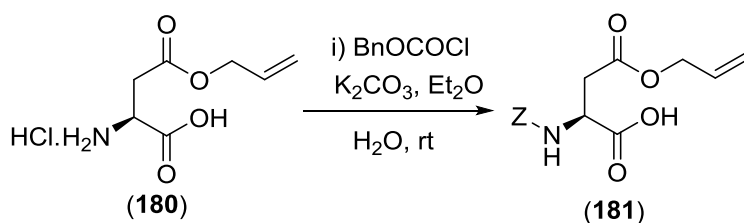


**2-((2S)-2-amino-3-carboxypropanoyl)amino)-4-oxo-4-(prop-2-en-1-yl-oxy)butanoic acid (179)**

**<sup>1</sup>H NMR** (D<sub>2</sub>O, 400 MHz)  $\delta$  3.13-3.15 (4H, m, 2 x 2H $\beta$ ), 4.37-4.40 (2H, m, 2 x H $\alpha$ ), 4.76 (2H, m, O-CH<sub>2</sub>-CH=CH<sub>2</sub>), 5.30-5.40 (2H, m, O-CH<sub>2</sub>-CH=CH<sub>2</sub>), 5.93-6.06 (1H, m, O-CH<sub>2</sub>-CH=CH<sub>2</sub>).

**Method B<sup>204</sup>:** Distilled allyl alcohol (50.0 mL, 735 mmol) was cooled to 0 °C, then acetyl chloride (7.0 mL, 99 mmol) was added dropwise to the reaction mixture and the resulting mixture was stirred at 0 °C for 1 h. Compound (**172**) (3.3 g, 25 mmol) was added to the reaction mixture and stirring continued at 0 °C, then the suspension was allowed to warm to room temperature overnight. After running the reaction for 18 h, the reaction mixture was cooled to 0 °C, then cold diethyl ether (250 mL) was added and the stirring continued at 0 °C for 1 h. The white precipitate was collected by filtration and washed with cold diethyl ether (2 x 20 mL) to give (**180**) as a white solid (4.3 g, 83%); **Mp** 170-174 °C (lit<sup>204</sup> 184-186 °C); **IR**  $\nu_{\max}$  (KBr disc): 3422 (NH), 2945 (CH), 2643 (OH), 1735 (C=O), 1503 (C=C), 1230 (CO) **<sup>1</sup>H NMR** (D<sub>2</sub>O, 400 MHz)  $\delta$  3.12-3.17 (2H, m, 2H $\beta$ ), 4.35-4.37 (1H, m, H $\alpha$ ), 4.70-4.74 (2H, m, O-CH<sub>2</sub>-CH=CH<sub>2</sub>), 5.30-5.40 (2H, m, O-CH<sub>2</sub>-CH=CH<sub>2</sub>), 5.93-6.02 (1H, m, O-CH<sub>2</sub>-CH=CH<sub>2</sub>).

**(2S)-4-(Allyloxy)-2-(benzyloxycarbonylamino)-4-oxobutanoic acid<sup>204</sup> (181)**

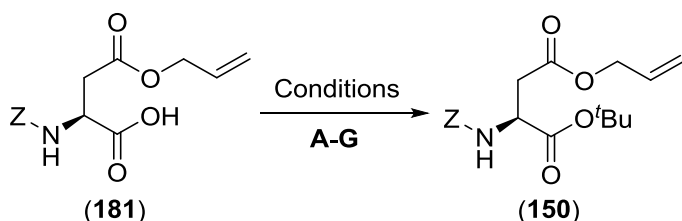


A stirred solution of (**180**) (4.0 g, 19 mmol) in H<sub>2</sub>O (90 mL) was treated with diethyl ether (40 mL), K<sub>2</sub>CO<sub>3</sub> (4.3 g, 31 mmol) and benzyl chloroformate (4.3 mL, 31 mmol), then the reaction mixture was stirred at room temperature for 16 h. The reaction was checked by TLC (10% MeOH/CH<sub>2</sub>Cl<sub>2</sub>) and a new spot with a higher R<sub>f</sub> appeared. The aqueous layer was extracted with diethyl ether (2 x 100 mL) and then it was acidified to pH 1 with concentrated aq. HCl.



The resulting opaque solution was extracted with diethyl ether (2 x 100 mL). The combined organic extracts were dried over MgSO<sub>4</sub>, filtered and the solvent was evaporated to give an oil. The crude material was dissolved in CH<sub>2</sub>Cl<sub>2</sub> and was purified by column chromatography using 7% MeOH/CH<sub>2</sub>Cl<sub>2</sub> eluent to give **(181)** as a colourless oil (3.3 g, 56%); **IR**  $\nu_{\text{max}}$  (thin film): 3337 (NH), 3068, 2951 (CH), 1727 (C=O), 1215 (CO); **<sup>1</sup>H NMR** (CDCl<sub>3</sub>, 400 MHz)  $\delta$  2.90 (1H, dd, *J* 17.2, 4.5 Hz, 1H $\beta$ ), 3.08 (1H, dd, *J* 17.3, 4.2 Hz, 1H $\beta$ ), 4.57-4.59 (2H, m, O-CH<sub>2</sub>-CH=CH<sub>2</sub>), 4.69-4.72 (1H, m, H $\alpha$ ), 5.12 (2H, s, CH<sub>2</sub>-Ph), 5.21-5.32 (2H, m, O-CH<sub>2</sub>-CH=CH<sub>2</sub>), 5.82-5.92 (2H, m, O-CH<sub>2</sub>-CH=CH<sub>2</sub>, NH), 7.30-7.35 (5H, m, Ph), 10.17 (1H, br, OH); **<sup>13</sup>C NMR** (CDCl<sub>3</sub>, 101 MHz)  $\delta$  36.37 (CH<sub>2</sub> $\beta$ ), 50.16 (CH $\alpha$ ), 65.81 (O-CH<sub>2</sub>-CH=CH<sub>2</sub>), 67.28 (O-CH<sub>2</sub>-Ph), 118.76 (CH=CH<sub>2</sub>), 128.05, 128.19, 128.48, (CH Ar), 131.47 (CH=CH<sub>2</sub>), 135.93 (C quaternary), 156.13, 170.63, 175.09 (C=O).

**(2S)-4-Allyl-1-tert-butyl-2-(((benzyloxy)carbonyl)amino)succinate (150)** <sup>204</sup>



**A:** Boc<sub>2</sub>O, CH<sub>3</sub>CN/*t*BuOH (v/v: 2/1), pyridine, DMAP, rt, 18 h; **B:** Boc<sub>2</sub>O, *t*BuOH, DMAP, rt, 16 h; **C:** IPCC, DMAP, *t*BuOH, Et<sub>3</sub>N, CH<sub>2</sub>Cl<sub>2</sub>, 35 °C, 16 h; **D:** as **C**, using only *t*BuOH, 16 h; **E:** *t*BuOH, DCC, DMAP, CH<sub>2</sub>Cl<sub>2</sub>, 0 °C to rt; **F:** *t*BuOH, HOBT, DCC, DMAP, DMF/CH<sub>2</sub>Cl<sub>2</sub>, rt, 16 h; **G:** as **B**, heating at 40 °C, 48 h.

**Method A:** A stirred solution of **(181)** (268 mg, 0.87 mmol) in acetonitrile/*t*BuOH (2:1, v/v) (60 mL) was treated with Boc<sub>2</sub>O (190 mg, 0.87 mmol), pyridine (70  $\mu$ L, 0.87 mmol) and DMAP (5.0 mg, 0.043 mmol) then the reaction mixture was stirred at room temperature for 18 h. The reaction mixture was checked by TLC (10% MeOH/CH<sub>2</sub>Cl<sub>2</sub>) which showed the starting material plus presence of a new spot with a higher R<sub>f</sub>. The solvents were evaporated and the residue was dissolved in EtOAc (50 mL) and then was extracted with sat. aq. NaHCO<sub>3</sub> (50 mL), 1M aq. HCl (2 x 50 mL) and brine (50 mL). The organic extract was dried over MgSO<sub>4</sub>, filtered and the solvent was evaporated to give an oil. The crude material was dissolved in CH<sub>2</sub>Cl<sub>2</sub> and then purified by column chromatography using 2% MeOH/CH<sub>2</sub>Cl<sub>2</sub> eluent to give **(150)** as an oil (59 mg, 19%).

**Method B:** A stirred solution of (**181**) (184 mg, 0.60 mmol) in *t*BuOH (6 mL, 0.06 mmol) was treated with Boc<sub>2</sub>O (258 mg, 1.18 mmol) and DMAP (5.0 mg, 0.043 mmol) then the reaction mixture was stirred at room temperature for 16 h. The reaction mixture was checked by TLC (10% MeOH/CH<sub>2</sub>Cl<sub>2</sub>) which showed the starting material plus the presence of a new spot with a higher R<sub>f</sub>. The reaction mixture was diluted in EtOAc (50 mL) and then was extracted with 5% aq. citric acid (2 x 20 mL), 5% aq. NaHCO<sub>3</sub> (2 x 20mL) and brine (20 mL). The organic extract was dried over MgSO<sub>4</sub>, filtered and the solvent was evaporated to give the desired compound (**150**) as an oil (52 mg, 24%).

**Method C:** A stirred solution of (**179**) (150 mg, 0.50 mmol) in distilled CH<sub>2</sub>Cl<sub>2</sub> (2 mL) was stirred at 35 °C and then triethylamine (74 µL, 0.54 mmol), DMAP (12 mg, 0.10 mmol), *t*BuOH (138 µL, 1.47 mmol) and IPCC (60 µL, 0.54 mmol) were added to the reaction mixture, which was stirred at 35 °C, for 16 h. The reaction mixture was checked by TLC (10% MeOH/CH<sub>2</sub>Cl<sub>2</sub>), which showed the starting material plus the presence of a new spot with a higher R<sub>f</sub>. All the solvents were evaporated. The residue was dissolved in EtOAc (30 mL) and then was extracted with 5% aq. citric acid (2 x 20 mL), 5% aq. NaHCO<sub>3</sub> (2 x 20mL) and brine (20 mL). The organic extract was dried over MgSO<sub>4</sub>, filtered and the solvent was evaporated to give the desired compound (**150**) as an oil (17 mg, 10%).

**Method D:** A stirred solution of (**181**) (150 mg, 0.50 mmol) in freshly distilled *t*BuOH (3 mL, 32 mmol) was stirred at 35 °C and then triethylamine (74 µL, 0.54 mmol), DMAP (12 mg, 0.10 mmol) and IPCC (60 µL, 0.54 mmol) were added to the reaction mixture and stirred at 35 °C, for 16 h. The reaction mixture was checked by TLC (10% MeOH/CH<sub>2</sub>Cl<sub>2</sub>), which showed the starting material plus the presence of a new spot with a higher R<sub>f</sub>. All the solvents were evaporated. The residue was dissolved in EtOAc (30 mL) and then was extracted with 5% aq. citric acid (2 x 20 mL), 5% aq. NaHCO<sub>3</sub> (2 x 20mL) and brine (20 mL). The organic extract was dried over MgSO<sub>4</sub>, filtered and the solvent was evaporated to give (**150**) as an oil (95 mg, 25%). Analysis by <sup>1</sup>H NMR showed the desired compound, plus the unchanged starting material.

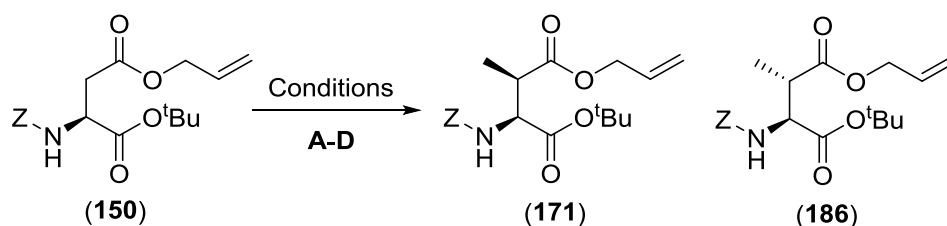
**Method E:** A stirred solution of DCC (121 mg, 0.60 mmol) and DMAP (6 mg, 0.05 mmol) in anhydrous CH<sub>2</sub>Cl<sub>2</sub> (5 mL) was cooled to 0 °C. *t*BuOH (460 µL, 4.9 mmol) and (**181**) (152 mg, 0.50 mmol) were added to the reaction mixture, which was then stirred at 0 °C for 1 h, then at 25 °C for 16 h. The reaction mixture was diluted in EtOAc (30 mL) and then was extracted with

5% aq. citric acid (2 x 20 mL), 5% aq. NaHCO<sub>3</sub> (2 x 20 mL) and brine (20 mL). The organic extract was dried over MgSO<sub>4</sub>, filtered and the solvent was evaporated to give a yellow solid. Analysis by <sup>1</sup>H NMR was not consistent with formation of the expected product.

**Method F:** A stirred solution of (**181**) (150 mg, 0.50 mmol) in DMF/CH<sub>2</sub>Cl<sub>2</sub> (1:1, v/v) (10 mL) was treated with DCC (101 mg, 0.50 mmol) and HOBt (93 mg, 0.69 mmol) and the resulting mixture was stirred at room temperature for 40 min. *t*BuOH (460 μL, 4.9 mmol) and DMAP (6 mg, 0.050 mmol) were added and then the reaction mixture was stirred at room temperature for 16 h. The reaction mixture was checked by TLC (10% MeOH/CH<sub>2</sub>Cl<sub>2</sub>), which showed the starting material plus the presence of a new spot with a higher R<sub>f</sub>. The reaction mixture was diluted in EtOAc (30 mL) and then was extracted with 5% aq. citric acid (2 x 20 mL), 5% aq. NaHCO<sub>3</sub> (2 x 20 mL) and brine (20 mL). The organic extract was dried over MgSO<sub>4</sub>, filtered and the solvent evaporated to give an oil. Analysis by <sup>1</sup>H NMR was not consistent with formation of the expected product.

**Method G:** A stirred solution of (**181**) (3.9 g, 0.013 mol) in freshly distilled *t*BuOH (95 mL, 1.01 mmol) was treated with Boc<sub>2</sub>O (5.6 g, 0.025 mmol) and DMAP (460 mg, 4x10<sup>-3</sup> mmol), then the reaction mixture was stirred at 40 °C for 48 h. The reaction mixture was checked by TLC (10% MeOH/CH<sub>2</sub>Cl<sub>2</sub>) which showed the disappearance of the starting material and the formation of a new spot with a higher R<sub>f</sub>. The solvents were evaporated. The residue was dissolved in EtOAc (100 mL) and was then extracted with 5% aq. citric acid (2 x 100 mL), 5% aq. NaHCO<sub>3</sub> (2 x 100 mL) and brine (100 mL). The organic extract was dried over MgSO<sub>4</sub>, filtered and the solvent was evaporated to give an oil. The crude product was dissolved in CH<sub>2</sub>Cl<sub>2</sub> and was purified by column chromatography using 1% MeOH/CH<sub>2</sub>Cl<sub>2</sub>, to give (**150**) (2.1 g, 45%) as a colourless oil; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz) δ 1.44 (9H, s, C(CH<sub>3</sub>)<sub>3</sub>), 2.81 (1H, dd, *J* 16.8, 4.4 Hz, 1Hβ), 3.00 (1H, dd, *J* 16.8, 4.4 Hz, 1Hβ), 4.49-4.60 (3H, m, O-CH<sub>2</sub>-CH=CH<sub>2</sub> and Hα), 5.11 (2H, s, CH<sub>2</sub>-Ph), 5.21-5.32 (2H, m, O-CH<sub>2</sub>-CH=CH<sub>2</sub>), 5.70-5.72 (1H, m, NH), 5.83-5.93 (1H, m, O-CH<sub>2</sub>-CH=CH<sub>2</sub>), 7.30-7.35 (5H, m, Ph); <sup>13</sup>C NMR (CDCl<sub>3</sub>, 101 MHz) δ 27.79 (C(CH<sub>3</sub>)<sub>3</sub>), 36.83 (CH<sub>2</sub>β), 50.91 (CHα), 65.48 (O-CH<sub>2</sub>-CH=CH<sub>2</sub>), 66.91 (O-CH<sub>2</sub>-Ph), 82.53 (C(CH<sub>3</sub>)<sub>3</sub>), 118.58 (CH=CH<sub>2</sub>), 127.99, 128.07, 128.44 (CH Ph), 131.68 (CH=CH<sub>2</sub>), 136.24 (C quaternary), 155.87 (C=O), 169.43 (C=O), 170.35 (C=O); [found (ES<sup>+</sup>) 386.1625 [M+Na]<sup>+</sup>, C<sub>19</sub>H<sub>25</sub>NO<sub>6</sub>Na requires 386.1574].

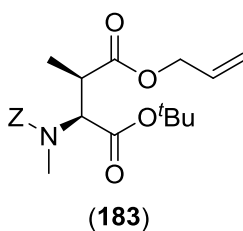
**(2*R*,3*S*)-1-Allyl 4-*tert*-butyl 3-(benzyloxycarbonylamino)-2-methylbutanedioate<sup>193</sup>(171)**



**A:** LiHMDS, THF, MeI, -78 °C to rt, 18 h; **B:** LiHMDS, THF, HMPA, MeI, -78 °C to rt, 18 h; **C:** as **B**, using anhydrous reagents; **D:** as **B**, at -78 °C for 3 h; **E:** as **A**, using anhydrous reagents and reaction at -78 °C, 3 h.

**Method A:** A stirred solution of (150) (320 mg, 0.88 mmol) in anhydrous THF (3 mL) under a N<sub>2</sub> atmosphere was cooled to -78 °C, then 1M LiHMDS in THF (1.8 mL, 1.8 mmol) was added to the reaction mixture and stirring was continued at -78 °C for 2 h. Iodomethane (66 µL, 1.05 mmol) was then added to the reaction mixture and the stirring was continued at -78 °C for 6 h, then the reaction was allowed to warm up to room temperature overnight. After running the reaction for 18 h, the reaction mixture was cooled to 0 °C and then it was quenched with cold 3 M aq. HCl to pH 4 and brine (5 mL) and was extracted with EtOAc (2 x 10 mL). The combined organic extracts were dried over MgSO<sub>4</sub>, filtered and the solvent was evaporated to give a yellow oil. The crude material was purified by column chromatography using 5% EtOAc/petroleum ether eluent to give (182) as a pale yellow oil (223 mg), a mixture of C- and N-alkylated products.

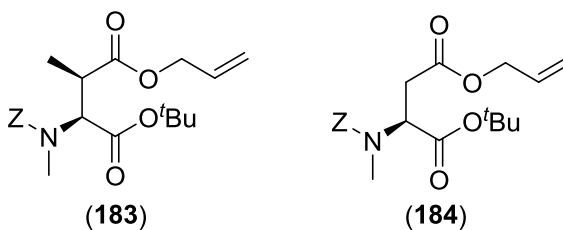
**Method B:** A stirred solution of (150) (320 mg, 0.88 mmol) in anhydrous THF (3 mL) under an N<sub>2</sub> atmosphere was cooled to -78 °C, then 1M LiHMDS in THF (1.8 mL, 1.8 mmol) and HMPA (0.95 mL, 5.46 mmol) were added to the reaction mixture and the resulting solution was allowed to stir at -78 °C for 45 min. Iodomethane (170 µL, 2.64 mmol) was added at -78 °C and the reaction mixture was allowed to warm up to room temperature overnight. After running the reaction for 18 h, a new spot with higher R<sub>f</sub> appeared, as shown by TLC (6% EtOAc/petroleum ether). Brine (20 mL) was added to the reaction mixture and then it was extracted with EtOAc (2 x 40 mL). The combined organic extracts were dried over MgSO<sub>4</sub>, filtered and the solvent was evaporated to give a brown oil. The crude material was purified by column chromatography using 6-7% EtOAc/petroleum ether eluent to give a pale yellow oil. Analysis by <sup>1</sup>H NMR revealed the following compounds:



**(2S, 3R)-4-Allyl 1-tert-butyl N-benzyloxycarbonyl-N,3-dimethyl-L-aspartate (183)**

An oil (117 mg, 40%), **R<sub>f</sub>** 0.41 TLC (10% EtOAc/petroleum ether); **IR**  $\nu_{\max}$  (thin film): 3451 (NH), 2978, 2942 (CH), 1734 (C=O), 1455, 1153 (CO); **<sup>1</sup>H NMR** (CDCl<sub>3</sub>, 400 MHz)  $\delta$  two rotamers: 1.24-1.28 (3H, m, CH(CH<sub>3</sub>)CO), 1.42 (4.5H, s, C(CH<sub>3</sub>)<sub>3</sub>), 1.43 (4.5H, s, C(CH<sub>3</sub>)<sub>3</sub>), 2.88-2.93 (3H, m, N(CH<sub>3</sub>)), 3.16-3.23 (1H, m, H $\beta$ ), 4.49-4.51 (2H, m, O-CH<sub>2</sub>-CH), 4.61-4.64 (0.5H, m, H $\alpha$ ), 4.81-4.84 (0.5H, m, H $\alpha$ ), 5.05-5.29 (4H, m, O-CH<sub>2</sub>-CH=CH<sub>2</sub>, CH<sub>2</sub>-Ph), 5.78-5.87 (1H, m, O-CH<sub>2</sub>-CH=CH<sub>2</sub>), 7.29-7.35 (5H, m, Ph); **<sup>13</sup>C NMR** (CDCl<sub>3</sub>, 101 MHz)  $\delta$  two rotamers: 14.39, 14.84 (CH<sub>3</sub>), 27.78, 27.81 (C(CH<sub>3</sub>)<sub>3</sub>), 31.87, 32.04 (NCH<sub>3</sub>), 40.26 (CH $\beta$ ), 61.68, 62.02 (CH $\alpha$ ), 65.32, 65.40 (O-CH<sub>2</sub>-CH=CH<sub>2</sub>), 67.34, 67.42 (O-CH<sub>2</sub>-Ph), 77.18 (C(CH<sub>3</sub>)<sub>3</sub>), 118.43, 118.63 (CH=CH<sub>2</sub>), 127.71, 127.87, 128.35 (CH Ar), 131.76, 131.86 (CH=CH<sub>2</sub>), 136.47, 136.64 (C quaternary), 155.85, 156.53, 168.29, 168.55 (C=O), 173.01, 173.09 (C=O ester); [found (ES<sup>+</sup>) 414.1881 [M+Na]<sup>+</sup>, C<sub>21</sub>H<sub>29</sub>NO<sub>6</sub>Na, requires 414.1887].

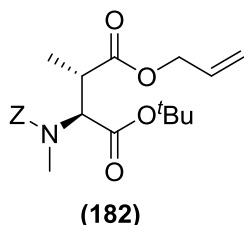
A mixture of **(183)** and N-alkylated product **(184)**, **R<sub>f</sub>** 0.40 TLC (10% EtOAc/petroleum ether) (22.4 mg).



**(2S)-1-tert-butyl-4-allyl-N-((benzyloxy)carbonyl)-N-methylaspartate (184)**

As an oil (56 mg, 17%); **IR**  $\nu_{\max}$  (thin film): 3452 (NH), 2976, 2934 (CH), 1734 (C=O), 1153 (CO); **<sup>1</sup>H NMR** (CDCl<sub>3</sub>, 400 MHz)  $\delta$  two rotamers: 1.37 (4.5H, s, C(CH<sub>3</sub>)<sub>3</sub>), 1.41 (4.5H, s, C(CH<sub>3</sub>)<sub>3</sub>), 2.69-2.83 (1H, m, H $\beta$ ), 2.94 (3H, s, N(CH<sub>3</sub>)), 2.96-3.11 (1H, m, H $\beta$ ), 4.57-4.62 (2H, m, O-CH<sub>2</sub>-CH), 4.74-4.80 (1H, m, CH $\alpha$ ), 5.05-5.34 (4H, m, O-CH<sub>2</sub>-CH=CH<sub>2</sub>, CH<sub>2</sub>-Ph), 5.83-5.93 (1H, m, O-CH<sub>2</sub>-CH=CH<sub>2</sub>), 7.29-7.34 (5H, m, Ph); **<sup>13</sup>C NMR** (CDCl<sub>3</sub>, 101 MHz)  $\delta$  two

rotamers: 27.78, 27.81, (C(CH<sub>3</sub>)<sub>3</sub>), 33.47, 33.59 (N-CH<sub>3</sub>), 34.64, 35.12 (CH<sub>2</sub>β), 57.69, 58.22 (CHα), 65.38, 65.48 (O-CH<sub>2</sub>=CH=CH<sub>2</sub>), 67.25, 67.45 (O-CH<sub>2</sub>-Ph), 82.06, 82.23 (C(CH<sub>3</sub>)<sub>3</sub>), 118.31, 118.49 (CH=CH<sub>2</sub>), 127.67, 127.76, 127.92, 128.39 (CH Ar), 131.76, 131.88 (CH=CH<sub>2</sub>), 136.29, 136.62 (C quaternary), 155.85, 156.16 (C=O), 168.89, 168.97 (C=O ester), 170.30, 170.54 (C=O ester); [found (ES<sup>+</sup>) 400.1784 [M+H]<sup>+</sup>, C<sub>20</sub>H<sub>28</sub>NO<sub>6</sub> requires 400.1731].



A mixture of both diastereoisomeric C/N-alkylated products **(182)** and **(183)** (41 mg, 12%)

**Method C:** A stirred solution of **(150)** (160 mg, 0.44 mmol), in anhydrous THF (2 mL) under a N<sub>2</sub> atmosphere was cooled to -78 °C. 1M LiHMDS in THF (0.92 mL, 0.92 mmol) and HMPA (0.47 mL, 2.73 mmol) were added to the reaction mixture and the resulting solution was allowed to stir at -78 °C for 45 min. Iodomethane (94 μL, 1.32 mmol) was added at -78 °C. After running the reaction for 3 h 30 min, the starting material was consumed and a new spot with higher R<sub>f</sub> appeared. The reaction was stopped by pouring into aqueous solution of 3M aq. HCl (15 mL) at room temperature and then the resulting mixture was extracted with EtOAc (3 x 20 mL). The combined organic extracts were dried over MgSO<sub>4</sub>, filtered and the solvent was evaporated to give a brown oil. The crude material was purified by column chromatography using 6% EtOAc/petroleum ether eluent to give a pale yellow oil. Analysis by <sup>1</sup>H NMR indicated both diastereoisomeric C/N-alkylated products **(182)** and **(183)** and N-alkylated product **(184)**.

**Method D:** A stirred solution of **(150)** (160 mg, 0.44 mmol), in anhydrous THF (2 mL) under a N<sub>2</sub> atmosphere was cooled to -78 °C. 1M LiHMDS in THF (0.92 mL, 0.92 mmol) and anhydrous HMPA (0.47 mL, 2.73 mmol) were added and the solution was allowed to stir at -78 °C for 45 min. Freshly distilled iodomethane (94 μL, 1.32 mmol) was added at -78 °C. The reaction was checked by TLC (8% EtOAc/petroleum ether) and after running the reaction for 3 h, the starting material was consumed and a new spot with higher R<sub>f</sub> appeared on TLC. The reaction was stopped by pouring into 3M aq. HCl (15 mL) at room temperature, and then the resulting mixture was extracted with EtOAc (4 x 20 mL). The combined organic extracts were dried over MgSO<sub>4</sub>, filtered and the solvent was evaporated to give a brown oil. The crude

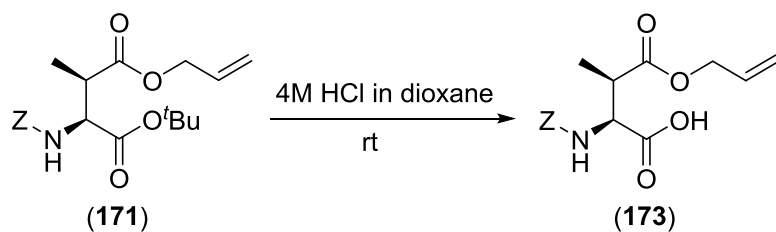
product was purified by column chromatography using 4-10% EtOAc/petroleum ether eluent to give a pale yellow oil. Analysis by  $^1\text{H}$  NMR indicated the desired C-alkylated product (**171a**) (92 mg, 55%) but contaminated with the C/N-alkylated N-alkylated products.

**Method E:** A stirred solution of (**150**) (600 mg, 1.65 mmol), in anhydrous THF (2 mL) under  $\text{N}_2$  atmosphere was cooled to  $-78^\circ\text{C}$ . 1M LiHMDS in THF (3.5 mL, 3.5 mmol) was added and the solution was allowed to stir at  $-78^\circ\text{C}$  for 45 min. Freshly distilled iodomethane (0.301 mL, 4.95 mmol) was added at  $-78^\circ\text{C}$ . After running the reaction for 3 h, the starting material was consumed and a new spot with higher  $R_f$  appeared on TLC. The reaction was stopped by pouring into 3M aq. HCl (50 mL) at room temperature and then the resulting mixture was extracted with EtOAc (4 x 20 mL). The combined organic extracts were dried over  $\text{MgSO}_4$ , filtered and the solvent was evaporated to give a brown oil. The crude material was purified by column chromatography using 8-9% EtOAc/petroleum ether eluent. This gave the desired compound C-alkylated product (**171**) (301 mg, 48%) as a pale yellow oil;  $[\alpha]^{22}_{\text{D}} = -6.2$  (c 1.1,  $\text{CHCl}_3$ ), (lit  $[\alpha]^{22}_{\text{D}} = -6.2$ , c 1.2,  $\text{CHCl}_3$ )<sup>193</sup>; **IR**  $\nu_{\text{max}}$  (thin film): 3433, 3368, (NH), 2960, 2941 (CH), 1729 (C=O), 1505 (C=C), 1218, 1160 (CO);  **$^1\text{H}$  NMR** ( $\text{CDCl}_3$ , 400 MHz)  $\delta$  1.21 (3H, d,  $J$  7.3 Hz,  $\text{CH}(\text{CH}_3)\text{CO}$ ), 1.42 (9H, s,  $\text{C}(\text{CH}_3)_3$ ), 3.18-3.23 (1H, m,  $\text{H}\beta$ ), 4.51-4.60 (3H, m,  $\text{O}-\text{CH}_2-\text{CH}$ ,  $\text{H}\alpha$ ), 5.12-5.33 (4H, m,  $\text{O}-\text{CH}_2-\text{CH}=\text{CH}_2$ ,  $\text{CH}_2-\text{Ph}$ ), 5.63-5.65 (1H, m,  $\text{NH}$ ), 5.84-5.94 (1H, m,  $\text{O}-\text{CH}_2-\text{CH}=\text{CH}_2$ ), 7.29-7.37 (5H, m, Ph);  **$^{13}\text{C}$  NMR** ( $\text{CDCl}_3$ , 101 MHz)  $\delta$  13.02 ( $\text{CH}_3$ ), 27.79 ( $\text{C}(\text{CH}_3)_3$ ), 41.43 ( $\text{CH}\beta$ ), 56.21 ( $\text{CH}\alpha$ ), 65.41 ( $\text{O}-\text{CH}_2-\text{CH}=\text{CH}_2$ ), 66.93 ( $\text{O}-\text{CH}_2-\text{Ph}$ ), 82.47 ( $\text{C}(\text{CH}_3)_3$ ), 118.31 ( $\text{CH}=\text{CH}_2$ ), 127.04, 128.15, 128.43 (CH Ar), 131.78 ( $\text{CH}=\text{CH}_2$ ), 136.30 (C quaternary), 156.44, 169.37 (C=O), 173.12 (C=O ester); [found (ES+) 400.1748  $[\text{M}+\text{H}]^+$ ,  $\text{C}_{20}\text{H}_{28}\text{NO}_6$  requires 400.1731].

A mixture of both diastereoisomeric C-alkylated products (**171**) and (**186**) plus the starting material (**150**) (200 mg, 33%) was also isolated.

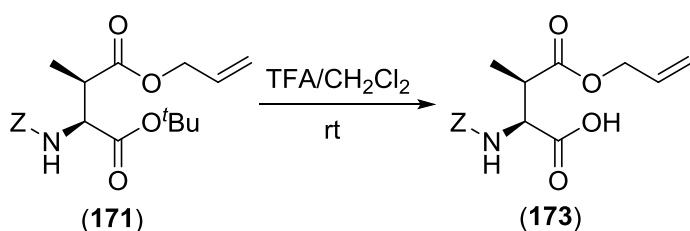
#### Preparation of (2*S*,3*R*)-4-(allyloxy)-2-((benzyloxy)carbonyl)amino)-3-methyl-4-oxobutanoic acid (**173**)<sup>185</sup>

##### Method A:



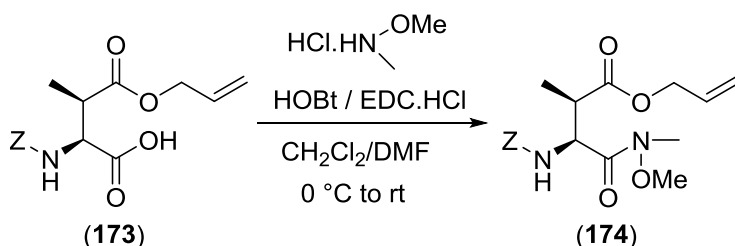
(**171**) (96 mg, 0.25 mmol) was treated with 4 M aq. HCl in dioxane (2 mL) at room temperature for 30 min. The reaction mixture was then co-evaporated with diethyl ether to give an oil. Analysis by  $^1\text{H}$  NMR showed the starting material.



**Method B:**

A stirred solution of **(171)** (228 mg, 0.60 mmol) in anhydrous  $\text{CH}_2\text{Cl}_2$  (60 mL) was treated with TFA (20 mL) and the stirring was continued at room temperature. The reaction was monitored by TLC and a new spot with a lower  $R_f$  0.60 (5% MeOH/ $\text{CH}_2\text{Cl}_2$  + 0.1% AcOH) appeared. After running the reaction for 2 h, the solvent was evaporated, to give **(173)** (176 mg, 91%) as an oil; **IR**  $\nu_{\text{max}}$  (thin film): 3342 (NH), 2946 (CH), 1717, 1703 (C=O), 1221 (CO);  **$^1\text{H}$  NMR** ( $\text{CDCl}_3$ , 400 MHz)  $\delta$  1.30 (3H, d,  $J$  7.4 Hz,  $\text{CH}_3(\text{CH})\text{CO}$ ), 3.26-3.36 (1H, m, H $\beta$ ), 4.53-4.68 (3H, m, O- $\text{CH}_2\text{-CH}$ , H $\alpha$ ), 5.10-5.32 (4H, m, O- $\text{CH}_2\text{-CH=CH}_2$ ,  $\text{CH}_2\text{-Ph}$ ), 5.70-5.99 (2H, m, O- $\text{CH}_2\text{-CH=CH}_2$ , NH), 7.15-7.38 (5H, m, Ph), 9.12 (1H, br, COOH);  **$^{13}\text{C}$  NMR** ( $\text{CDCl}_3$ , 101 MHz)  $\delta$  13.73 (CH $_3\text{-CH}\beta$ ), 41.16 (CH $\beta$ ), 55.50 (CH $\alpha$ ), 65.83 (O- $\text{CH}_2\text{-CH=CH}_2$ ), 67.32 (O- $\text{CH}_2\text{-Ph}$ ), 118.76 (CH=CH $_2$ ), 127.99, 128.18, 128.49 (CH Ph), 131.40 (CH=CH $_2$ ), 135.97 (C quaternary), 156.77 (C=O), 173.79, 175.13 (C=O ester); [found (ES $^+$ ) 344.1124 [M+Na] $^+$ , C $_{16}\text{H}_{19}\text{NO}_6\text{Na}$  requires 344.1101].

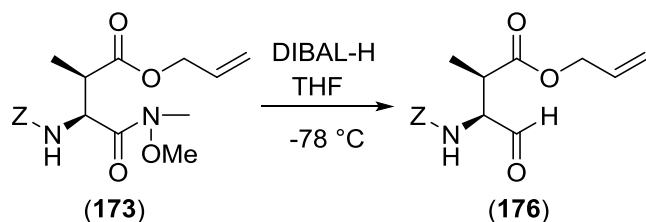
**(2R,3S)-Allyl-3-((benzyloxy)carbonyl)amino)-4-(methoxy(methyl)amino)-2-methyl-4-oxobutanoate (174)**



A stirred solution of **(173)** (207 mg, 0.65 mmol) in anhydrous  $\text{CH}_2\text{Cl}_2$  (2.5 mL) and DMF (2.5 mL) was cooled to 0  $^\circ\text{C}$  and HOBT (123 mg, 0.91 mmol) and EDC.HCl (125 mg, 0.65 mmol) were added and the stirring continued at 0  $^\circ\text{C}$  for 30 min. *N,O*-Dimethylhydroxylamine hydrochloride (63 mg, 0.65 mmol) and DIPEA (0.6 mL, 3.4 mmol) were added to the reaction mixture. The resulting solution was allowed to warm up to room temperature overnight. After

running the reaction for 16 h, TLC showed a new spot with a lower  $R_f$  0.50 (15% EtOAc/petroleum ether). All the solvent was evaporated and the residue was dissolved in EtOAc (20 mL) and extracted with 5% aq. citric acid (2 x 20 mL), 5% aq.  $\text{NaHCO}_3$  (2 x 20 mL) and brine (20 mL). The organic layer was dried over  $\text{MgSO}_4$ , filtered and the solvent was evaporated to give an oil. The crude material was purified by column chromatography using 90% EtOAc/petroleum ether eluent to give (**174**) as oil (127 mg, 53%);  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400 MHz)  $\delta$  1.21 (3H, d,  $J$  7.1 Hz,  $\text{CH}(\text{CH}_3)\text{CO}$ ), 2.90-3.02 (1H, m,  $\text{H}_\beta$ ), 3.20 (3H, s,  $\text{NCH}_3$ ), 3.77 (3H, s,  $\text{OCH}_3$ ) 4.50-4.60 (2H, m,  $\text{O-CH}_2\text{-CH}_2\text{=CH}$ ), 4.90-4.94 (1H, m,  $\text{H}_\alpha$ ), 5.05-5.32 (4H, m,  $\text{O-CH}_2\text{-CH=CH}_2$ ,  $\text{CH}_2\text{-Ph}$ ), 5.78-5.92 (2H, m,  $\text{O-CH}_2\text{-CH=CH}_2$  and  $\text{NH}$ ), 7.26-7.37 (5H, m, Ph);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 101 MHz)  $\delta$  13.99 ( $\text{CH}(\text{CH}_3)\text{CO}$ ), 32.20 ( $\text{NCH}_3$ ), 41.57 ( $\text{CH}_\beta(\text{CH}_3)\text{CO}$ ), 53.07 ( $\text{CH}_\alpha$ ), 61.60 ( $\text{OCH}_3$ ), 65.48 ( $\text{O-CH}_2\text{-CH}_2\text{=CH}$ ), 66.90 ( $\text{CH}_2\text{-Ar}$ ), 118.33 ( $\text{O-CH}_2\text{-CH}_2\text{=CH}$ ), 127.92, 128.03, 128.41 ( $\text{CH Ph}$ ), 131.91 ( $\text{O-CH}_2\text{-CH}_2\text{=CH}$ ), 136.21 (C quaternary), 156.15 (C=O ester), 173.21 (C=O amide); [found (ES $^+$ ) 387.1517  $[\text{M}+\text{Na}]^+$ ,  $\text{C}_{18}\text{H}_{24}\text{N}_2\text{O}_6\text{Na}$  requires 387.1527].

#### Attempted preparation of (2*R*,3*S*)-allyl-3-(((benzyloxy)carbonyl)amino)-2-methyl-4-oxobutanoate (**176**)

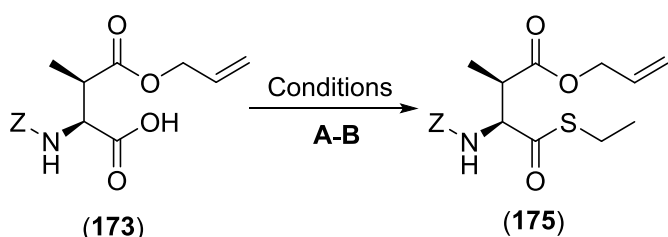


#### *Via reduction of Weinreb amide*

A stirred solution of (**173**) (124 mg, 0.34 mmol) in anhydrous THF (4.3 mL) under  $\text{N}_2$  was cooled to  $-78\text{ }^\circ\text{C}$ , then DIBAL-H (0.41 mL, 0.41 mmol) was added and the stirring continued at  $-78\text{ }^\circ\text{C}$ . The reaction was monitored by TLC (35% EtOAc/petroleum ether) and after running the reaction for 2 h, the starting material remained and another 1.2 eq of DIBAL-H (0.41 mL, 0.41 mmol) was added. After 2 h reaction, 0.85 eq of DIBAL-H (0.29 mL, 0.29 mmol) was added to the reaction mixture. After running the reaction for 9 h, the reaction mixture was poured into 5% aq. citric acid (10 mL) and then extracted with EtOAc (3 x 40 mL). The combined organic layers were dried over  $\text{Na}_2\text{SO}_4$ , filtered and the solvent evaporated to give

an oil (238 mg). Analysis by  $^1\text{H}$  NMR showed the presence of the aldehyde (**176**). The crude material was then dissolved in MeOH and pre-absorbed onto silica, then purified by column chromatography using EtOAc/petroleum ether eluent, plus 0.1% pyridine. The desired compound (**176**) could not be separated from the starting material (**174**).

**(2*R*,3*S*)-Allyl-3-(((benzyloxy)carbonyl)amino)-4-(ethylthio)-2-methyl-4-oxobutanoate**  
**(175)**



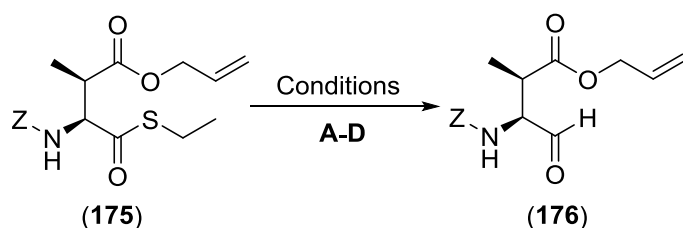
**A:** EtSH, DCC, DMAP,  $\text{CH}_2\text{Cl}_2$ , rt, 16 h; **B:** EtSH, EDC.HCl, DMAP, rt, 16 h.

**Method A:** A stirred solution of (**173**) (138 mg, 0.43 mmol) in anhydrous  $\text{CH}_2\text{Cl}_2$  (10 mL), was treated with DCC (97 mg, 0.47 mmol). EtSH (333  $\mu\text{L}$ , 0.43 mmol) (0.96  $\mu\text{L}$  of EtSH dissolved in 1 mL of anhydrous  $\text{CH}_2\text{Cl}_2$ ) and DMAP (5.2 mg, 0.043 mmol) were added to the reaction mixture. The resulting mixture was allowed to stir at room temperature for 16 h. After consumption of the starting material as shown by TLC (30% EtOAc/petroleum ether), the solvent was evaporated. The residue was dissolved in EtOAc (30 mL), then extracted with 5% aq. citric acid (2 x 20 mL), 5% aq.  $\text{NaHCO}_3$  (2 x 20 mL) and brine (20 mL). The organic layer was dried over  $\text{Na}_2\text{SO}_4$ , filtered and the solvent was evaporated to give an oil. The crude material was purified by column chromatography using 20% EtOAc/petroleum ether eluent to give (**175**) (81 mg, 52%) as an oil,  $[\alpha]^{22}_{\text{D}} = -0.90$  (c 1.4,  $\text{CHCl}_3$ ).  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400 MHz)  $\delta$  1.22 (3H, t,  $J$  7.4 Hz,  $\text{CH}_3\text{-CH}_2$ ), 1.26 (3H, d,  $J$  7.6 Hz,  $\text{CH}(\text{CH}_3)\text{CO}$ ), 2.81-2.90 (2H, m,  $\text{CH}_3\text{-CH}_2\text{-S}$ ), 3.37-3.44 (1H, m,  $\text{H}\beta$ ), 4.50-4.60 (3H, m,  $\text{O-CH}_2\text{-CH}$ ,  $\text{H}\alpha$ ), 5.12-5.33 (4H, m,  $\text{O-CH}_2\text{-CH=CH}_2$ ,  $\text{CH}_2\text{-Ph}$ ), 5.81-5.98 (2H, m,  $\text{O-CH}_2\text{-CH=CH}_2$ ,  $\text{NH}$ ), 7.30-7.39 (5H, m, Ph);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 101 MHz)  $\delta$  14.18 ( $\text{CH}(\text{CH}_3)\text{CO}$ ), 23.41 ( $\text{CH}_3\text{-CH}_2$ ), 27.80 ( $\text{CH}_3\text{-CH}_2$ ), 40.72 ( $\text{CH}\beta(\text{CH}_3)\text{CO}$ ), 62.63 ( $\text{CH}\alpha$ ), 65.56 ( $\text{O-CH}_2\text{-CH}_2\text{=CH}$ ), 67.30 ( $\text{CH}_2\text{-Ph}$ ), 118.52 ( $\text{O-CH}_2\text{-CH}_2\text{=CH}$ ), 127.93, 128.15, 128.47 ( $\text{CH Ph}$ ), 131.52 ( $\text{O-CH}_2\text{-CH}_2\text{=CH}$ ), 136.15 (C quaternary), 156.15 (C=O ester), 173.21 (C=O amide), 200.37 (C=O thiol ester); [found (ES $^+$ ) 387.1170  $[\text{M}+\text{Na}]^+$ ,  $\text{C}_{18}\text{H}_{23}\text{NO}_5\text{SNa}$  requires 387.1189].

**Method B:** A stirred solution of (**173**) (178 mg, 0.55 mmol) in peptide grade DMF (3 mL), was treated with EDC.HCl (94 mg, 0.60 mmol). EtSH (441  $\mu$ L, 0.55 mmol) and DMAP (9.0 mg, 55  $\mu$ mol) were added to the reaction mixture. The resulting mixture was allowed to stir at room temperature for 16 h. After consumption of the starting material as showed on TLC (30% EtOAc/petroleum ether) the solvent was evaporated. The residue was dissolved in EtOAc (30 mL), then was extracted with 5% aq. citric acid (2 x 20 mL), 5% aq. NaHCO<sub>3</sub> (2 x 20 mL) and brine (20 mL). The organic layer was dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and the solvent was evaporated to give an oil. The crude product was purified by column chromatography using 10-12% EtOAc/petroleum ether eluent to give (**175**) 81 mg, 40%) as an oil.

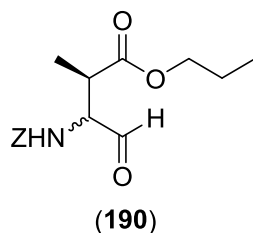
**Attempted preparation of (2*R*,3*S*)-allyl 3-(benzyloxycarbonylamino)-2-methyl-4-oxobutanoate (**176**)**

*Via selective reduction of a thiol ester*



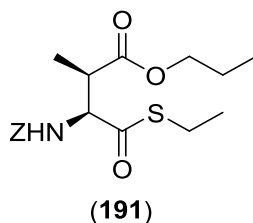
**A:** Pd/BaSO<sub>4</sub>, Et<sub>3</sub>SiH, acetone, rt, 48 h; **B:** as **A**, reaction for 2 h; **C:** 5%Pd/CaCO<sub>3</sub>/PbO, quinoline, 1-decene, Et<sub>3</sub>SiH, acetone, 4 h; **D:** Pd/BaSO<sub>4</sub>, 1-decene, Et<sub>3</sub>SiH, 2 h.

**Method A:** A stirred solution of (**175**) (72 mg, 0.20 mmol) in distilled acetone (2 mL) was treated with 20% w/w Pd/BaSO<sub>4</sub> (17 mg, 20) and triethylsilane (160  $\mu$ L, 0.98 mmol) and then the stirring was continued at room temperature for 48 h. After consumption of the starting material, three new spots appeared by TLC (30% EtOAc/petroleum ether). The reaction mixture was filtered through Celite and then washed with acetone (10 mL) to give an oil (86 mg). The crude material was purified by column chromatography using 50% EtOAc/petroleum ether eluent, to give (3.4 mg, 7%) of the following epimerized aldehyde:



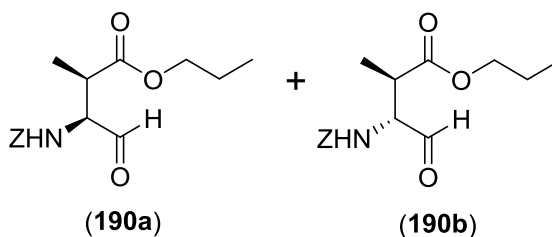
**(3S)-Propyl-3-((benzyloxy)carbonylamino)-2-methyl-4-carbaldehyde (190)**

**Method B:** A stirred solution of (**175**) (44 mg, 0.12 mmol) in distilled acetone (1.5 mL), was treated with 20% Pd/BaSO<sub>4</sub> (9 mg) and triethylsilane (96  $\mu$ L, 0.60 mmol) and then the stirring was continued at room temperature. The reaction was monitored by TLC (20% EtOAc/petroleum ether). After running the reaction for 2 h 15min, all the starting material was consumed and then the reaction mixture was filtered through Celite, then washed with acetone (10 mL) to give an oil (69 mg). The crude material was purified by column chromatography using 5-100% EtOAc/petroleum ether eluent, to give the following compounds:



**Propyl (2R,3S)-3-(benzyloxycarbonylamino)4-(ethylthio)-2-methyl-4-oxobutanoate (191)** (13.4 mg, 30%)

**<sup>1</sup>H NMR** (CDCl<sub>3</sub>, 400 MHz)  $\delta$  0.91 (3H, t,  $J$  7.4 Hz, CH<sub>3</sub>-CH<sub>2</sub>), 1.16-1.28 (6H, m, CH<sub>3</sub>-CH<sub>2</sub> and CH(CH<sub>3</sub>)CO), 1.54-1.68 (2H, m, CH<sub>3</sub>-CH<sub>2</sub>-CH<sub>2</sub>), 2.81-2.90 (2H, m, CH<sub>3</sub>-CH<sub>2</sub>-S), 3.35-3.41 (1H, m, H $\beta$ ), 3.97-4.07 (2H, m, O-CH<sub>2</sub>-CH<sub>2</sub>), 4.50 (1H, dd,  $J$  10.2, 3.6 Hz, H $\alpha$ ), 5.19 (2H, s, CH<sub>2</sub>-Ph), 5.97-5.99 (1H, m, NH), 7.31-7.40 (5H, m, Ph); [found (ES<sup>+</sup>) 390.1329 [M+Na]<sup>+</sup>, C<sub>18</sub>H<sub>25</sub>NO<sub>5</sub>SNa requires 390.1346].



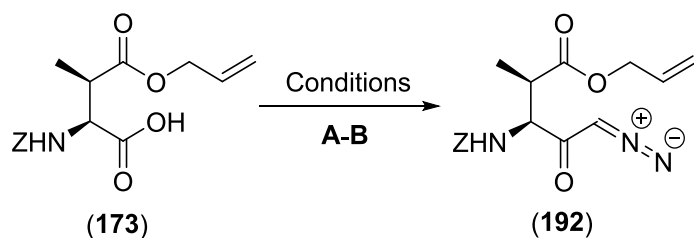
**(2R,3S)-propyl-3-((benzyloxy)carbonylamino)-2-methyl-4-oxobutanoate (190a) plus its epimer (190b)** (13 mg, 35%).

**<sup>1</sup>H NMR** (CDCl<sub>3</sub>, 400 MHz)  $\delta$  0.92 (3H, td, *J* 1.6, 7.4 Hz, CH<sub>3</sub>-CH<sub>2</sub>), 1.29 (1.5H, d, *J* 7.4 Hz, CH(CH<sub>3</sub>)CO), 1.34 (1.5H, d, *J* 7.4 Hz, CH(CH<sub>3</sub>)CO), 1.57-1.70 (2H, m, CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>3</sub>), 3.05-3.08 (0.45H, m, H $\beta$ ), 3.29-3.35 (0.55H, m, H $\beta$ ), 3.91-4.11 (2H, m, O-CH<sub>2</sub>-CH<sub>2</sub>), 4.38-4.41 (1H, m, H $\alpha$ ), 4.43-4.46 (1H, m, H $\alpha$ ), 5.12 (1H, s, CH<sub>2</sub>-Ph), 5.19 (1H, s, CH<sub>2</sub>-Ph), 5.60-5.64 (0.45H, m, NH), 5.83-5.86 (0.55H, m, NH), 7.30-7.38 (5H, m, Ph), 9.66 (0.5H, s, HC=O), 9.70 (0.5H, s, HC=O).

**Method C:** A stirred solution of (**175**) (37 mg, 0.10 mmol) in distilled acetone (2 mL), was treated with 5% Pd/CaCO<sub>3</sub>/PbO (403 mg), a few drops of quinoline and 1-decene (382  $\mu$ L, 2.02 mmol) and then the reaction mixture was stirred vigorously at room temperature. Triethylsilane (40  $\mu$ L, 0.25 mmol) was added over 2 min and the stirring continued at room temperature. The reaction was monitored by TLC (20% EtOAc/petroleum ether). After running the reaction for 1 h 40 min, 3 drops of triethylsilane were added to the reaction mixture in order to complete the reaction. After running the reaction for 4 h a new spot with lower R<sub>f</sub> appeared and then the reaction mixture was filtered through Celite, which was washed with acetone to give an oil (146 mg). The crude material was purified by column chromatography using 5-100% EtOAc/petroleum ether eluent, to give only the unchanged (**175**) (30 mg).

**Method D:** A stirred solution of (**175**) (14 mg, 0.038 mmol) in distilled acetone (1 mL), was treated with 20% w/w Pd/BaSO<sub>4</sub> (7 mg, 20%) and 1-decene (382  $\mu$ L, 2.02 mmol) and then the reaction mixture was stirred at room temperature. Triethylsilane (40  $\mu$ L, 0.25 mmol) was added to the reaction, which was stirred vigorously at room temperature. The reaction was monitored by TLC (20% EtOAc/petroleum ether). After running the reaction for 2 h, the starting material was consumed. The reaction mixture was filtered through cotton wool, which was washed with acetone to give an oil (15 mg). The crude product was analysed by <sup>1</sup>H NMR showing the presence of (**190a**) and (**191**).

**(2*R*,3*S*)-Allyl-3-((benzyloxy)carbonyl)amino-5-diazo-2-methyl-4-oxopentanoate (192)**



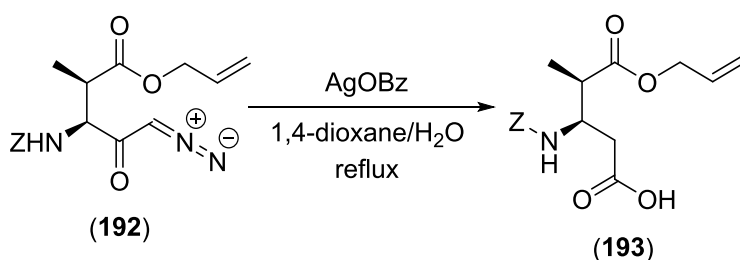
**A:** TBTU, DIPEA, THF/DMF, 0 °C, then TMSCHN<sub>2</sub>, 3 h 30 min; **B:** isopropyl chloroformate, DIPEA, THF, -15 °C to -5 °C, then TMSCHN<sub>2</sub>, 4 °C, 48 h.

**Method A:** A stirred solution of **(173)** (307 mg, 0.96 mmol) in THF (3 mL) and DMF (2 mL) was cooled to 0 °C and was treated with distilled DIPEA (170 µL, 0.96 mmol), TBTU (308 mg, 0.96 mmol) and the stirring was continued at 0 °C for 30 min. 2M TMSCHN<sub>2</sub> in Et<sub>2</sub>O (0.96 mL, 1.92 mmol) was added to the reaction mixture, and the resulting solution was stirred at 0 °C. The reaction was monitored by TLC (1% MeOH/CH<sub>2</sub>Cl<sub>2</sub> + 0.1% AcOH). After running the reaction for 3 h 30 min, the starting material was consumed. The reaction mixture was diluted in EtOAc (150 mL) then extracted with 5% aq. citric acid (20 mL), 5% aq. NaHCO<sub>3</sub> (20 mL) and brine (20 mL). The organic layer was dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and the solvent was evaporated to give a brown oil (452 mg) 1. Analysis by <sup>1</sup>H NMR of the crude product showed the lack of a H $\beta$  signal.

**Method B:** A stirred solution of **(173)** (196 mg, 0.61 mmol) in anhydrous THF (3 mL), under a N<sub>2</sub> atmosphere was treated with DIPEA (109 µL, 0.63 mmol), then the reaction mixture was cooled to -15 °C. The resulting mixture was treated with isobutyl chloroformate (82 µL, 0.63 mmol) and the stirring continued at -5 °C for 30 min. The white precipitated solid was filtered off and the filtrate was diluted in acetonitrile (2 mL) and treated with 2M TMSCHN<sub>2</sub> in Et<sub>2</sub>O (0.61 mL, 1.22 mmol) and stirring continued at 4 °C, for 48 h. TLC (4% MeOH/CH<sub>2</sub>Cl<sub>2</sub>) showed the disappearance of the starting material and formation a new spot at higher R<sub>f</sub>. The reaction mixture was diluted in EtOAc (40 mL), then extracted with 5% aq. citric acid (20 mL), 5% aq. NaHCO<sub>3</sub> (20 mL) and brine (20 mL). The organic layer was dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and the solvents evaporated to give **(192)** (170 mg, 83%) as a brown oil. Analysis by <sup>1</sup>H NMR of the crude product showed the desired compound which was used directly in the next step. <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz)  $\delta$  1.23 (3H, d, *J* 7.4 Hz, CH(CH<sub>3</sub>)CO)), 3.28-3.33 (1H, m, H $\beta$ ),

4.39-4.52 (1H, m, H $\alpha$ ), 4.54-4.67 (2H, m, O-CH<sub>2</sub>-CH=CH<sub>2</sub>), 5.15 (2H, s, CH<sub>2</sub>-Ph), 5.21-5.35 (2H, m, O-CH<sub>2</sub>-CH=CH<sub>2</sub>), 5.59 (1H, s, CH=N=N), 5.82-5.92 (1H, m, O-CH<sub>2</sub>-CH=CH<sub>2</sub>), 6.02-6.05 (1H, m, NH), 7.31-7.37 (5H, m, Ph).

**(3*R*,4*R*)-3-(Benzyloxycarbonylamino)-4-methyl-5-oxo-5-allyloxypentanoic acid (193)**

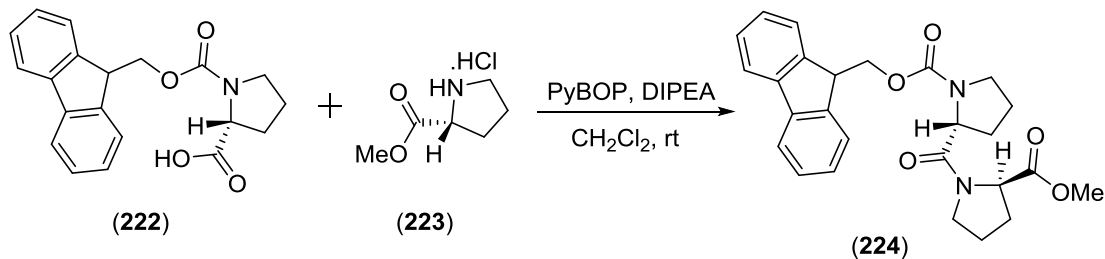


A stirred solution of diazomethylketone (**192**) (67 mg, 0.19 mmol) in 1,4-dioxane (2 mL) and H<sub>2</sub>O (1 mL), was treated with silver benzoate (7.0 mg, 0.03 mmol) and then the reaction mixture was stirred at 70 °C, for 48 h. The reaction was checked by TLC (4% MeOH/CH<sub>2</sub>Cl<sub>2</sub>) which showed the disappearance of the starting material and the formation of a new spot at lower R<sub>f</sub>. The reaction mixture was then diluted in 5% aq. NaHCO<sub>3</sub> (10 mL), then it was extracted with Et<sub>2</sub>O (20 mL). The aqueous layer was then acidified with saturated aq. citric acid to pH 2 and it was extracted with EtOAc (3 x 30 mL) to give a colourless oil (109 mg). The crude material was pre-adsorbed into silica and purified by column chromatography using 1% MeOH/CH<sub>2</sub>Cl<sub>2</sub> eluent to give (**193**) (19 mg, 27%); <sup>1</sup>H NMR (CD<sub>3</sub>OD, 400 MHz)  $\delta$  1.24 (3H, d, *J* 7.6 Hz, CH(CH<sub>3</sub>)CO), 2.53-2.66 (2H, m, CH<sub>2</sub>-COOH), 2.82-2.94 (1H, m, CH-CHNHZ), 4.27-4.35 (1H, m, CH-CHNHZ), 4.57-4.66 (2H, m, O-CH<sub>2</sub>-CH=CH<sub>2</sub>), 5.09-5.39 (4H, m, O-CH<sub>2</sub>-CH=CH<sub>2</sub>, CH<sub>2</sub>-Ph), 5.93-6.03 (1H, m, O-CH<sub>2</sub>-CH=CH<sub>2</sub>), 7.33-7.41 (5H, m, Ph); <sup>13</sup>C NMR (CD<sub>3</sub>OD, 101 MHz)  $\delta$  14.09 (CH(CH<sub>3</sub>)CO), 38.09 (CH), 44.63 (CH<sub>2</sub>-CO<sub>2</sub>H), 66.36 (O-CH<sub>2</sub>-CH=CH<sub>2</sub> and CH<sub>2</sub>-Ph), 67.48 (CH), 118.52 (O-CH<sub>2</sub>-CH=CH<sub>2</sub>), 128.76, 128.97, 129.48 (CH Ar), 133.61 (O-CH<sub>2</sub>-CH=CH<sub>2</sub>), 138.36 (C quaternary), 158.13 (C=O acid), 175.55 (C=O ester); [found (ES<sup>+</sup>) 386.1273 [M+Na]<sup>+</sup>, C<sub>18</sub>H<sub>21</sub>NO<sub>7</sub>Na requires 386.1261].



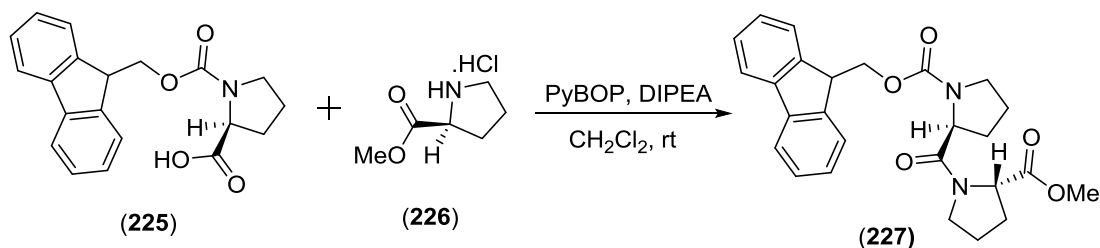
## 7.5. Synthesis of Diketopiperazines

### Fmoc-D-Pro-D-Pro-OMe (**224**)



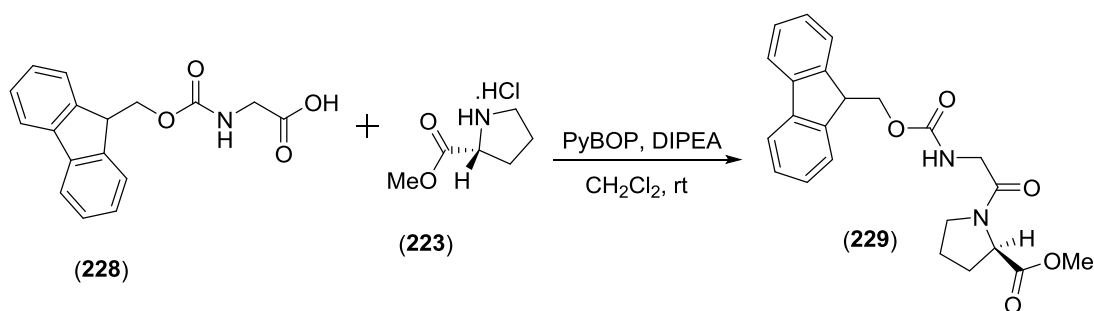
Fmoc-D-Pro-OH (**222**) (507 mg, 1.50 mmol), H-D-Pro-OMe.HCl (**223**) (249 mg, 1.50 mmol) and PyBOP (785 mg, 1.50 mmol) were dissolved in anhydrous CH<sub>2</sub>Cl<sub>2</sub> (5 mL). The reaction mixture was treated with DIPEA (0.8 mL, 4.5 mmol) and then stirred at room temperature overnight when the starting material was consumed, as shown by TLC (10% MeOH/CH<sub>2</sub>Cl<sub>2</sub>). The reaction mixture was concentrated under vacuum, and the residue dissolved in EtOAc (20 mL) and extracted with 5% aq. citric acid (2 x 20 mL), sat. aq. NaHCO<sub>3</sub> (2 x 20 mL) and brine (2 x 20 mL). The organic extract was dried, filtered and the solvent was evaporated to give a yellow oil. The crude material was pre-adsorbed onto silica using CH<sub>2</sub>Cl<sub>2</sub> as a solvent and purified by column chromatography (80-90% EtOAc/petroleum ether eluent) to give (**224**) as a colourless oil (399 mg, 59%) (lit<sup>261</sup> Mp 118-120 °C); RP-HPLC (analytical system, gradient 1) R<sub>t</sub> 6.3 min; IR  $\nu_{\text{max}}$  (thin film) 3279 (NH), 3009, 2981, 2956, 2882 (CH), 1743, 1699, 1658 (C=O), 1433 (Ar, NH), 1355, 1191, 1123 (CO), 752 (CH Ar); <sup>1</sup>H NMR (270 MHz, CDCl<sub>3</sub>) two rotamers  $\delta$  1.91-2.20 (8H, m, 4 x H $\beta$  Pro, 4 x H $\gamma$  Pro), 3.44-3.63 (4H, m, 4 x H $\delta$  Pro), 3.68 (1.5H, s, CO<sub>2</sub>Me), 3.70 (1.5H, s, CO<sub>2</sub>Me), 4.15-4.30 (2H, m, Fmoc-CH<sub>2</sub>), 4.33-4.41 (1H, m, Fmoc-CH), 4.45-4.60 (2H, m, 2 x H $\alpha$  Pro), 7.24-7.40 (4H, m, Ar-H Fmoc), 7.51-7.63 (2H, m, Ar-H Fmoc), 7.66-7.75 (2H, m, Ar-H Fmoc); <sup>13</sup>C NMR (CDCl<sub>3</sub>, 101 MHz)  $\delta$  22.90, 24.14, 24.72, 24.74, 24.83 (2 x CH<sub>2</sub> $\gamma$  Pro), 28.45, 28.62, 28.94, 29.81 (2 x CH<sub>2</sub> $\beta$  Pro), 45.97, 46.45, 46.64, 47.06 (2 x CH<sub>2</sub>  $\delta$  Pro), 47.09 (CH Fmoc), 52.01 (OCH<sub>3</sub>), 58.01, 58.61 (2 x CH $\alpha$  Pro), 67.28 (CH<sub>2</sub> Fmoc), 119.59, 119.77, 124.99, 125.14, 126.87, 126.91, 127.49 (CH Ar), 141.12, 143.36 (C quaternary), 154.81 (C=O), 170.79 (C=O), 172.45 (C=O ester); [found (ES<sup>+</sup>) 449.1942 [M+H]<sup>+</sup>, C<sub>26</sub>H<sub>29</sub>N<sub>2</sub>O<sub>2</sub> requires 449.2071].

### Fmoc-L-Pro-L-Pro-OMe (**227**)

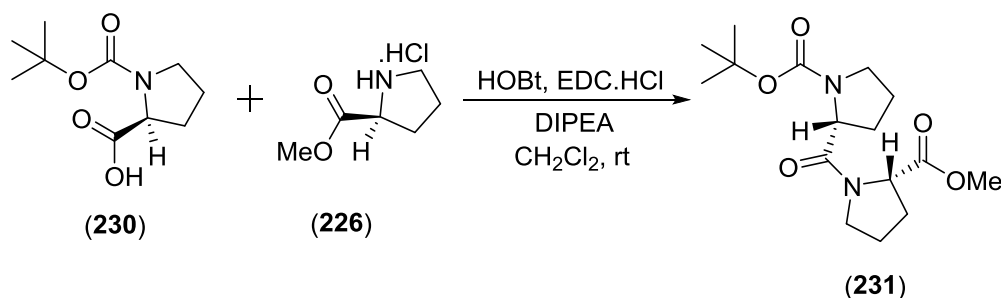


Fmoc-L-Pro-OH (**225**) (507 mg, 1.50 mmol), (**226**) (249 mg, 1.50 mmol) and PyBOP (785 mg, 1.50 mmol), were dissolved in anhydrous CH<sub>2</sub>Cl<sub>2</sub> (5 mL). The reaction mixture was treated with DIPEA (0.8 mL, 4.5 mmol) and then stirred at room temperature overnight when the starting material Fmoc-L-Pro-OMe was consumed by TLC (10% MeOH/CH<sub>2</sub>Cl<sub>2</sub>). The reaction mixture was concentrated under vacuum and the residue dissolved in EtOAc (20 mL) and extracted with 5% aq. citric acid (2 x 40 mL), sat. aq. NaHCO<sub>3</sub> (2 x 40 mL) and brine (2 x 40 mL). The organic extract was dried, filtered and the solvent was evaporated to give a yellow oil. The crude material was dissolved in CH<sub>2</sub>Cl<sub>2</sub> and purified by column chromatography (4% MeOH/CH<sub>2</sub>Cl<sub>2</sub> eluent) to give (**227**) as a colourless oil, which crystallised on standing (342 mg, 51%), (lit 118-120 °C); <sup>1</sup>H NMR (CDCl<sub>3</sub>, 270 MHz) two rotamers δ 1.70-2.25 (8H, m, 4 x H<sub>β</sub> Pro, 4 x H<sub>γ</sub> Pro), 3.47-3.55 (4H, m, 4 x H<sub>δ</sub> Pro), 3.63 (1.5H, s, CO<sub>2</sub>Me), 3.65 (1.5H, s, CO<sub>2</sub>Me), 4.11-4.35 (3H, m, Fmoc-CH<sub>2</sub>, Fmoc-CH), 4.44-4.50 (2H, m, 2 x H<sub>α</sub> Pro), 7.21-7.34 (4H, m, Ar-H Fmoc), 7.46-7.59 (2H, m, Ar-H Fmoc), 7.66 (2H, t, *J* 5.8 Hz, Ar-H Fmoc).

### Fmoc-Gly-D-Pro-OMe (**229**)

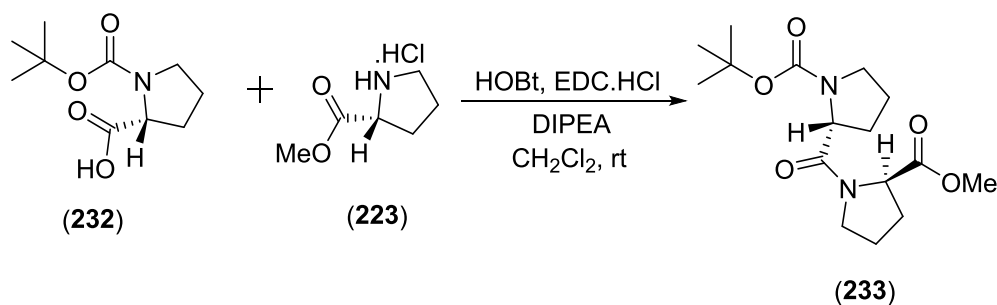


Fmoc-Gly-OH (**228**) (446 mg, 1.50 mmol), HOBt (283 mg, 2.10 mmol) and EDC.HCl (287 mg, 1.50 mmol) were dissolved in anhydrous CH<sub>2</sub>Cl<sub>2</sub> (5 mL) and DMF (5 mL). The resulting solution was stirred for 30 min at 0 °C, then (**223**) (249 mg, 1.50 mmol) and DIPEA (0.5 mL, 3 mmol) were added and the resulting mixture was allowed to warm gradually to room temperature overnight. The reaction mixture was concentrated under vacuum and the residue dissolved in EtOAc (20 mL) and extracted with 5% aq. citric acid (2 x 40 mL), sat. aq. NaHCO<sub>3</sub> (2 x 40 mL) and brine (2 x 40 mL). The organic extract was dried, filtered and the solvent was evaporated to give an oil. The crude material was purified by column chromatography (4% MeOH/CH<sub>2</sub>Cl<sub>2</sub> eluent) to give (**229**) as a colourless oil which crystallised on standing (347 mg, 57%); **Mp** 82-85°C; **IR**  $\nu_{\text{max}}$  (thin film) 3359 (NH), 3016, 2955, 2884 (CH), 1727, 1651 (C=O), 1447 (Ar, NH), 1223, 1178 (CO), 754 (CH Ar); **<sup>1</sup>H NMR** (CDCl<sub>3</sub>, 270 MHz)  $\delta$  1.84-2.20 (4H, m, 2 x H $\beta$  Pro, 2 x H $\gamma$  Pro), 3.38-3.64 (2H, m, 2 x H $\delta$  Pro), 3.70 (3H, s, CO<sub>2</sub>Me), 3.99 (2H, t, *J* 4.4 Hz, Fmoc-CH<sub>2</sub>), 4.15-4.20 (1H, m, Fmoc-CH), 4.31 (2H, d, *J* 7.1 Hz, CH<sub>2</sub> $\alpha$  Gly), 4.43-4.54 (1 H, m, H $\alpha$  Pro), 5.80-5.86 (1 H, m, NH), 7.25 (2H, t, *J* 7.3 Hz, Ar-H Fmoc), 7.35 (2H, t, *J* 7.3 Hz, Ar-H Fmoc), 7.57 (2H, d, *J* 7.4 Hz, Ar-H Fmoc), 7.71 (2H, d, *J* 7.1 Hz, Ar-H Fmoc); **<sup>13</sup>C NMR** (CDCl<sub>3</sub>, 101 MHz)  $\delta$  24.62 (CH<sub>2</sub> $\gamma$  Pro), 29.00 (CH<sub>2</sub> $\beta$  Pro), 43.35 (CH<sub>2</sub> $\delta$  Pro), 45.93, (CH<sub>2</sub> $\alpha$  Gly) 47.08 (CH Fmoc), 52.41 (OCH<sub>3</sub>), 58.93 (CH $\alpha$  Pro), 67.19 (CH<sub>2</sub> Fmoc), 119.93, 125.16, 126.59, 127.07 (CH Ar), 141.26, 143.87 (C quaternary), 156.23 (C=O), 167.02 (C=O), 172.27 (C=O ester); [found (ES<sup>+</sup>) 409.1761 [M+H]<sup>+</sup>, C<sub>23</sub>H<sub>25</sub>N<sub>2</sub>O<sub>5</sub> requires 409.1763].

**Boc-L-Pro-L-Pro-OMe (231)**<sup>256</sup>

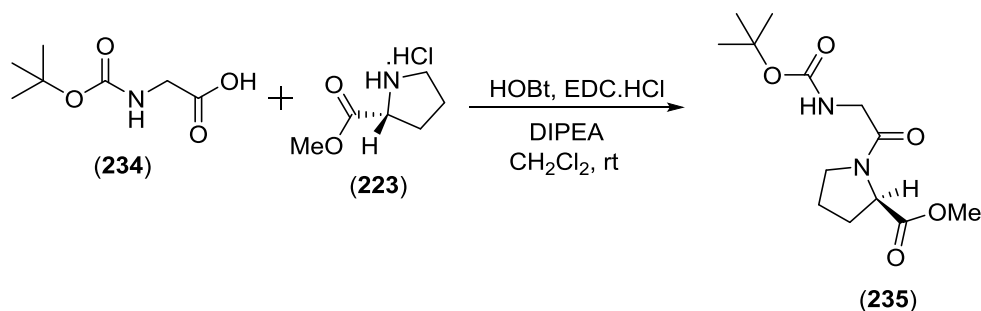
Boc-L-Pro-OH (**230**) (323 mg, 1.50 mmol), HOBT (283 mg, 2.10 mmol) and EDC.HCl (287 mg, 1.50 mmol) were dissolved in anhydrous CH<sub>2</sub>Cl<sub>2</sub> (5 mL). The resulting solution was allowed to stir for 30 min at 0 °C. H-L-Pro-OMe.HCl (**226**) (249 mg, 1.5 mmol) and DIPEA (0.52 mL, 3 mmol) were added and the resulting mixture was allowed to warm gradually to room temperature overnight. The reaction mixture was concentrated under vacuum and the residue dissolved in EtOAc (20 mL) and extracted with 5% aq. citric acid (2 x 40 mL), sat. aq. NaHCO<sub>3</sub> (2 x 40 mL) and brine (2 x 40 mL). The organic extract was dried, filtered and the solvent was evaporated to give an oil. The crude material was purified by column chromatography using 1-5% MeOH/CH<sub>2</sub>Cl<sub>2</sub> eluent, to give (**231**) as oil (292 mg, 60%); <sup>1</sup>H NMR (CDCl<sub>3</sub>, 270 MHz) δ two rotamers 1.35 (3.5H, s, C(CH<sub>3</sub>)<sub>3</sub> minor rotamer), 1.41 (5.5H, s, C(CH<sub>3</sub>)<sub>3</sub> major rotamer), 1.79-2.18 (8H, m, 4 x H<sub>γ</sub> Pro, 4 x H<sub>β</sub> Pro), 3.32-3.61 (3.4H, m, 3.4 x H<sub>δ</sub> Pro both rotamers), 3.66 (1.7H, s, OMe major rotamer), 3.68 (1.3H, s, OMe minor rotamer), 3.70-3.74 (0.6H, m, 0.6 x H<sub>δ</sub> Pro major rotamer), 4.36 (0.4H, dd, *J* 8.0, 4.0 Hz, 0.4 x H<sub>α</sub> Pro minor rotamer), 4.47 (0.6H, dd, *J* 8.0, 4.0 Hz, 0.6 x H<sub>α</sub> Pro major rotamer), 4.49-4.56 (1H, m, H<sub>α</sub> Pro both rotamers).

### Boc-D-Pro-D-Pro-OMe (**233**)



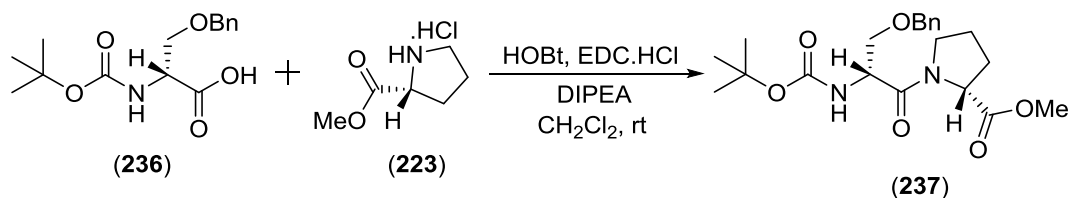
Boc-D-Pro-OH (**232**) (323 mg, 1.50 mmol), HOBT (283 mg, 2.10 mmol) and EDC.HCl (287 mg, 1.50 mmol) were dissolved in anhydrous CH<sub>2</sub>Cl<sub>2</sub> (5 mL). The resulting solution was allowed to stir for 30 min at 0 °C. Compound (**223**) (249 mg, 1.50 mmol) and DIPEA (0.52 mL, 3.0 mmol) were added and the resulting mixture was allowed to warm gradually to room temperature overnight. The reaction mixture was concentrated under vacuum and the residue dissolved in EtOAc (20 mL) and extracted with 5% aq. citric acid (2 x 40 mL), sat. aq. NaHCO<sub>3</sub> (2 x 40 mL) and brine (2 x 40 mL). The organic extract was dried, filtered and the solvent was evaporated to give an oil. The crude material was purified by column chromatography using 1-3% MeOH/CH<sub>2</sub>Cl<sub>2</sub> eluent, to give (**233**) as an oil (252 mg, 51%); <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz) δ two rotamers 1.33 (3.7H, s, C(CH<sub>3</sub>)<sub>3</sub> minor rotamer), 1.39 (5.3H, s, C(CH<sub>3</sub>)<sub>3</sub> major rotamer), 1.75-2.16 (8H, m, 4 x H<sub>γ</sub> Pro, 4 x H<sub>β</sub> Pro), 3.32-3.59 (3.7H, m, 3.7 x H<sub>δ</sub> Pro both rotamers), 3.64 (1.7H, s, OMe major rotamer), 3.66 (1.3H, s, OMe minor rotamer), 3.71-3.73 (0.3H, m, 0.3 x H<sub>δ</sub>Pro minor rotamer), 4.32-4.35 (0.4H, dd, *J* 8.0, 4.0 Hz, 0.4 x H<sub>α</sub> Pro minor rotamer), 4.43-4.46 (1H, dd, *J* 8.0, 4.0 Hz, H<sub>α</sub> Pro both rotamers), 4.47 (0.6H, m, 0.6 x CH<sub>α</sub>-Pro major rotamer).

### Boc-Gly-D-Pro-OMe (235)



Boc-Gly-OH (**234**) (263 mg, 1.50 mmol), HOBt (284 mg, 2.10 mmol) and EDC.HCl (288 mg, 1.50 mmol) were dissolved in anhydrous CH<sub>2</sub>Cl<sub>2</sub> (5 mL). The resulting solution was allowed to stir for 30 min at 0 °C. Compound (**223**) (249 mg, 1.50 mmol) and DIPEA (0.52 mL, 3.0 mmol) were added and the resulting mixture was allowed to warm gradually to room temperature overnight. The reaction mixture was concentrated under vacuum and the residue dissolved in EtOAc (20 mL) and extracted with 5% aq. citric acid (2 x 40 mL), sat. aq. NaHCO<sub>3</sub> (2 x 40 mL) and brine (2 x 40 mL). The organic extract was dried, filtered and the solvent was evaporated to give an oil. The crude material was purified by column chromatography using 1-4% MeOH/CH<sub>2</sub>Cl<sub>2</sub> eluent, to give (**235**) as oil (180 mg, 42%) (lit<sup>257</sup> Mp 55.9 °C); **IR**  $\nu_{\text{max}}$  (thin film) 3399 (NH), 2977, 2884 (CH), 1746, 1715, 1659 (C=O), 1443 (NH), 1170 (CO); **<sup>1</sup>H NMR** (CDCl<sub>3</sub>, 400 MHz)  $\delta$  1.39 (9H, s, C(CH<sub>3</sub>)<sub>3</sub>), 1.87-2.10 (4H, m, 2 x H $\beta$  Pro, 2 x H $\gamma$  Pro), 3.39-3.45 (1H, m, 1 x H $\delta$  Pro), 3.52-3.58 (1H, m, 1 x H $\delta$  Pro), 3.68 (3H, s, OMe), 3.87-3.93 (2H, m, CH<sub>2</sub> $\alpha$  Gly), 4.46-4.49 (1H, m, H $\alpha$  Pro), 5.37 (1H, br, NH).

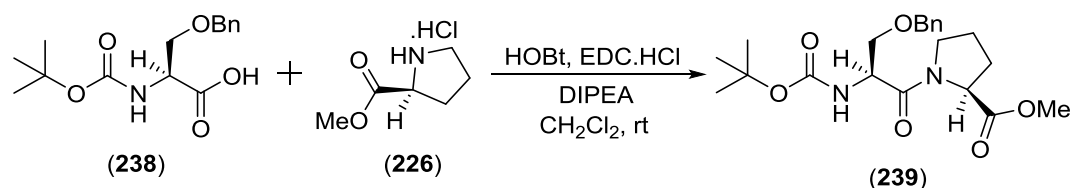
### Boc-D-Ser(OBn)-D-Pro-OMe (237)



Boc-D-Ser(OBn)-OH (**236**) (444 mg, 1.50 mmol), HOBt (283 mg, 2.10 mmol) and EDC.HCl (287 mg, 1.50 mmol) were dissolved in anhydrous CH<sub>2</sub>Cl<sub>2</sub> (5 mL) and DMF (5 mL). The

resulting solution was allowed to stir for 30 min at 0 °C. Compound (**223**) (249 mg, 1.5 mmol) and DIPEA (0.5 mL, 3 mmol) were added and the resulting mixture was allowed to warm gradually to room temperature overnight. The reaction mixture was concentrated under vacuum and the residue was dissolved in EtOAc (20 mL) and extracted with 5% aq. citric acid (2 x 40 mL), sat. aq. NaHCO<sub>3</sub> (2 x 40 mL) and brine (2 x 40 mL). The organic extract was dried, filtered and the solvent was evaporated to give an oil. The crude material was purified by column chromatography (4% MeOH/CH<sub>2</sub>Cl<sub>2</sub> eluent) to give (**237**) as an oil (304 mg, 75%); <sup>1</sup>H NMR (CDCl<sub>3</sub>, 270 MHz) two rotamers δ 1.35 (9H, s, C(CH<sub>3</sub>)<sub>3</sub>), 1.89-2.15 (3H, m, 2 x H<sub>γ</sub> Pro, 1 x H<sub>β</sub> Pro), 3.36-3.72 (7H, m, 1 x H<sub>β</sub> Pro, 2 x H<sub>δ</sub> Pro, 1 x H<sub>β</sub> Ser, CO<sub>2</sub>Me), 4.41-4.58 (4H, m, 1 x H<sub>β</sub> Ser, H<sub>α</sub> Ser, CH<sub>2</sub>Ph), 4.68 (0.8H, q, *J* 6.1 Hz, 0.8 x H<sub>α</sub> Pro major rotamer) 4.74 (0.2H, dd, *J* 8.0, 1.9 Hz, 0.2 x H<sub>α</sub> Pro minor rotamer) 5.32 (0.2H, d, *J* 8.6 Hz, 0.2 x NH minor rotamer), 5.40 (0.8H, d, *J* 8.5 Hz, 0.8 x NH major rotamer), 7.21-7.27 (5H, m, Ph).

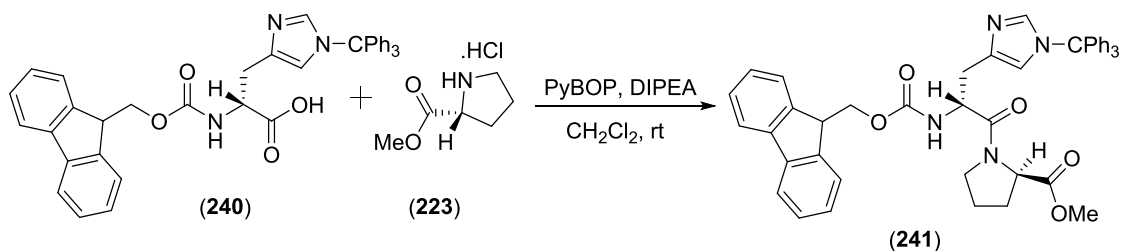
#### Boc-L-Ser(OBn)-L-Pro-OMe (**239**)<sup>262,263</sup>



Boc-L-Ser(OBn)-OH (**238**) (443 mg, 1.50 mmol), HOBT (284 mg, 2.10 mmol) and EDC·HCl (288 mg, 1.50 mmol) were dissolved in anhydrous CH<sub>2</sub>Cl<sub>2</sub> (5 mL) and DMF (5 mL). The resulting solution was allowed to stir for 30 min at 0 °C. Compound (**226**) (249 mg, 1.50 mmol) and DIPEA (0.52 mL, 3.0 mmol) were added and the resulting mixture was allowed to warm gradually to room temperature overnight. The reaction mixture was concentrated under vacuum and the residue dissolved in EtOAc (20 mL) and extracted with 5% aq. citric acid (2 x 40 mL), sat. aq. NaHCO<sub>3</sub> (2 x 40 mL) and brine (2 x 40 mL). The organic extract was dried, filtered and the solvent was evaporated to give an oil. The crude material was purified by column chromatography to give (**239**) as an oil (507 mg, 83%) (lit Mp ; IR ν<sub>max</sub> (thin film) 3317 (NH), 2975, 2877 (CH), 1747, 1710, 1649 (C=O), 1446 (NH), 1170 (CO); <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz) δ two rotamers 1.41 (2.14H, s, C(CH<sub>3</sub>)<sub>3</sub> minor rotamer), 1.42 (6.86H, s, C(CH<sub>3</sub>)<sub>3</sub> major rotamer), 1.93-2.26 (3 H, m, 2 x H<sub>γ</sub> Pro, 1 x H<sub>β</sub> Pro), 3.61-3.72 (7H, m, 1 x H<sub>β</sub> Pro, 2 x H<sub>δ</sub> Pro, 1 x H<sub>β</sub> Ser, CO<sub>2</sub>Me), 4.46-4.70 (5H, m, 1 x H<sub>β</sub> Ser, CH<sub>2</sub>Ph, H<sub>α</sub> Pro, H<sub>α</sub> Ser), 5.35 (1H, d, *J* 8.0 Hz, NH), 7.23-7.34 (5H, m, Ph); <sup>13</sup>C NMR (CDCl<sub>3</sub>, 101 MHz) δ two rotamers 24.80, 22.26

(CH<sub>2</sub>γ Pro), 28.22, 28.27 (C(CH<sub>3</sub>)<sub>3</sub>), 28.95, 30.65 (CH<sub>2</sub>β Pro), 46.37, 46.98 (CH<sub>2</sub>δ Pro), 51.97, 52.07 (CH<sub>2</sub>β Ser), 58.93 (CH<sub>α</sub> Ser), 59.06 (CH<sub>α</sub> Pro), 70.49, 73.23 (PhCH<sub>2</sub>-O), 79.55, 79.68 (C(CH<sub>3</sub>)<sub>3</sub>), 127.39, 127.45, 127.64, 128.25 (CH Ar), 137.79, 137.91 (C quaternary), 154.72, 155.29, 169.36, 172.19 (C=O).

#### Fmoc-D-His(Trt)-D-Pro-OMe (**241**)

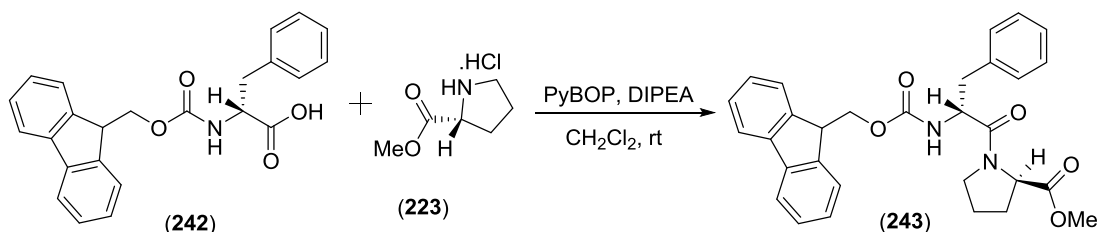


Compound (**240**) (309 mg, 0.500 mmol), (**223**) (83 mg, 0.50 mmol) and PyBOP (260 mg, 0.500 mmol) were dissolved in anhydrous CH<sub>2</sub>Cl<sub>2</sub> (7 mL). The reaction mixture was treated with DIPEA (0.17 mL, 2.0 mmol) and the reaction mixture was stirred at room temperature until the starting material was consumed, as shown by TLC (5% MeOH/CH<sub>2</sub>Cl<sub>2</sub>). The reaction mixture was concentrated under vacuum and the residue dissolved in EtOAc (20 mL) and extracted with 5% aq. citric acid (3 x 40 mL), sat. aq. NaHCO<sub>3</sub> (3 x 40 mL) and brine (3 x 40 mL). The organic extract was dried, filtered and the solvent was evaporated to give a yellow oil. The crude material was purified by column chromatography (4% MeOH/CH<sub>2</sub>Cl<sub>2</sub> eluent) to give (**241**) as a white solid (348 mg, 95%); **Mp** 115-120°C; **IR**  $\nu_{\text{max}}$  (KBr disc) 3412 (NH), 3060, 3020 (CH), 1715, 1494, 1447 (Ar, NH), 1213 (CO), 751 (CH Ar). **<sup>1</sup>H NMR** (CDCl<sub>3</sub>, 270 MHz)  $\delta$  1.89-2.12 (3H, m, 2 x H<sub>γ</sub> Pro, 1 x H<sub>β</sub> Pro), 2.83-2.91 (2H, m, 1 x H<sub>β</sub> Pro, 1 x H<sub>β</sub>-His), 3.06-3.13 (1H, dd, *J* 14.7 Hz, 4.8 Hz, 1 x H<sub>β</sub> His), 3.51 (3H, s, CO<sub>2</sub>Me), 3.63-3.79 (2H, m, 2 x H<sub>δ</sub> Pro), 4.12-4.20 (1H, m, Fmoc-CH), 4.25 (2H, t, *J* 6.5 Hz, Fmoc-CH<sub>2</sub>), 4.54-4.56 (1H, m, H<sub>α</sub> Pro), 4.79-4.87 (1H, m, H<sub>α</sub> His), 6.06 (1H, d, *J* 8.3 Hz, NH), 6.73 (1H, s, imidazole ring), 7.09-7.13 (6H, m, Ar-H), 7.22-7.37 (13H, m, Ar-H, 1 x imidazole ring), 7.57 (2H, d, *J* 5.6 Hz, Ar-H Fmoc), 7.73 (2H, d, *J* 7.4 Hz, Ar-H Fmoc); **<sup>13</sup>C NMR** (CDCl<sub>3</sub>, 101 MHz)  $\delta$  24.85 (CH<sub>2</sub>γ Pro, CH<sub>2</sub>γ His), 28.97 (CH<sub>2</sub>β Pro), 31.10 (CH<sub>2</sub>δ Pro), 46.91, (CH<sub>2</sub>β His) 47.12 (CH Fmoc), 52.09 (OCH<sub>3</sub>), 52.70 (CH<sub>α</sub> Pro), 58.68 (CH<sub>α</sub> His), 67.11 (CH<sub>2</sub> Fmoc), 119.86, 125.31, 127.04, 127.96, 128.03, 129.86 (CH Ar), 136.06 (CH imidazole ring), 138.63 (C=N), 141.20, 142.48,



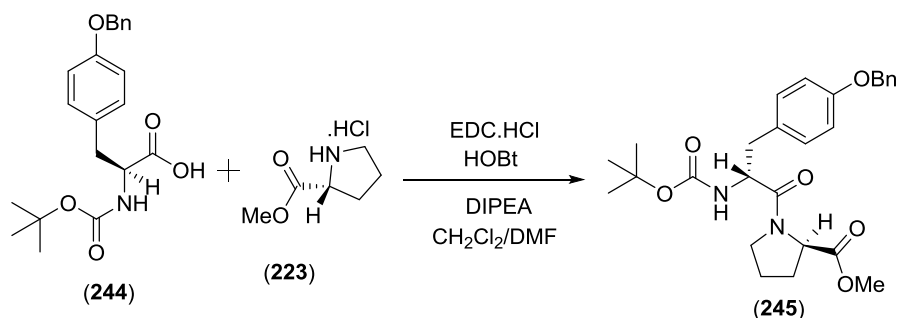
143.97, 143.99, (C quaternary), 156.05, 170.50 (C=O), 172.40 (C=O); [found (ES<sup>+</sup>) 731.3166 [M+H]<sup>+</sup>, C<sub>46</sub>H<sub>43</sub>N<sub>4</sub>O<sub>5</sub> requires 731.3228].

### Fmoc-D-Phe-D-Pro-OMe (**243**)



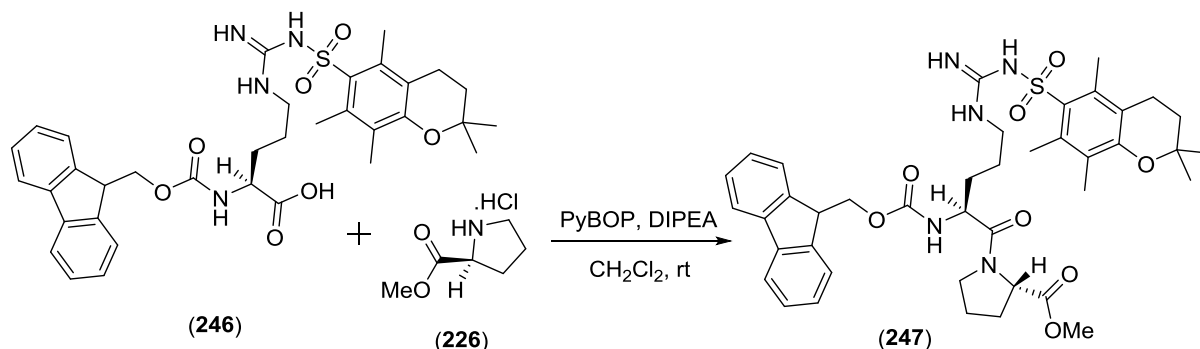
Compound (**242**) (390 mg, 1.00 mmol), (**223**) (167 mg, 1.00 mmol) and PyBOP (520 mg, 1.00 mmol), were dissolved in anhydrous CH<sub>2</sub>Cl<sub>2</sub> (7 mL). The reaction mixture was treated with DIPEA (0.39 mL, 2.0 mmol) and the reaction mixture was stirred at room temperature overnight, until the disappearance of the starting material, Fmoc-D-Phe-OH, was shown by TLC (10% MeOH/CH<sub>2</sub>Cl<sub>2</sub>). The reaction mixture was evaporated to dryness and the residue was dissolved in EtOAc (20 mL) and extracted with 5% aq. citric acid (3 x 40 mL), sat. aq. NaHCO<sub>3</sub> (3 x 40 mL) and brine (3 x 40 mL). The organic extract was filtered, dried and the solvent was evaporated to give a yellow oil. The crude material was purified by column chromatography (6-8% MeOH/CH<sub>2</sub>Cl<sub>2</sub> eluent) to give (**243**) as a colourless oil (393 mg, 79%); **IR**  $\nu_{\text{max}}$  (thin film) 3287 (NH), 2953, (CH), 1717, 1640, 1658 (C=O), 1443 (Ar, NH), 1250 (CO), 748 (CH Ar); **<sup>1</sup>H NMR** (CDCl<sub>3</sub>, 270 MHz)  $\delta$  1.67-2.15 (3H, m, 1 x H $\beta$  Pro, 2 x H $\gamma$  Pro), 2.99-3.01 (1H, m, 1 x H $\beta$  Pro), 3.06-3.23 (2H, m, 2 x H $\delta$  Pro), 3.57-3.60 (1H, m, 1 x H $\beta$  Phe), 3.75 (3H, s, CO<sub>2</sub>Me), 4.15-4.34 (4H, m, Fmoc-CH, Fmoc-CH<sub>2</sub>, 1 x H $\beta$  Phe), 4.48-4.52 (1H, m, H $\alpha$  Pro), 4.72 (1H, q, *J* 7.6 Hz, H $\alpha$  Phe), 5.69-5.75 (1H, m, NH), 7.25-7.31 (9H, m, Ar-H Fmoc, Ph, Phe), 7.51-7.53 (2H, m, Ar-H Fmoc), 7.74 (2H, d, *J* 7.4 Hz, Ar-H Fmoc); **<sup>13</sup>C NMR** (CDCl<sub>3</sub>, 101 MHz)  $\delta$  24.89 (CH<sub>2</sub> $\gamma$  Pro), 29.01 (CH<sub>2</sub> $\beta$  Pro), 39.03 (CH<sub>2</sub> $\delta$  Pro), 46.92 (CH<sub>2</sub> $\beta$  Phe), 47.08 (CH Fmoc), 52.24 (OCH<sub>3</sub>), 53.72 (CH $\alpha$  Pro), 59.00 (CH $\alpha$  Phe), 67.06 (CH<sub>2</sub> Fmoc), 119.94, 125.21, 126.94, 127.67, 128.59, 129.39 (CH Ar), 136.06, 141.26, 143.81 (C quaternary), 155.69, 170.32 (C=O), 172.21 (C=O); [found (ES<sup>+</sup>) 499.2239 [M+H]<sup>+</sup>, C<sub>30</sub>H<sub>31</sub>N<sub>2</sub>O<sub>5</sub> requires 499.2227].

### Boc-D-Tyr(OBn)-D-Pro (245)



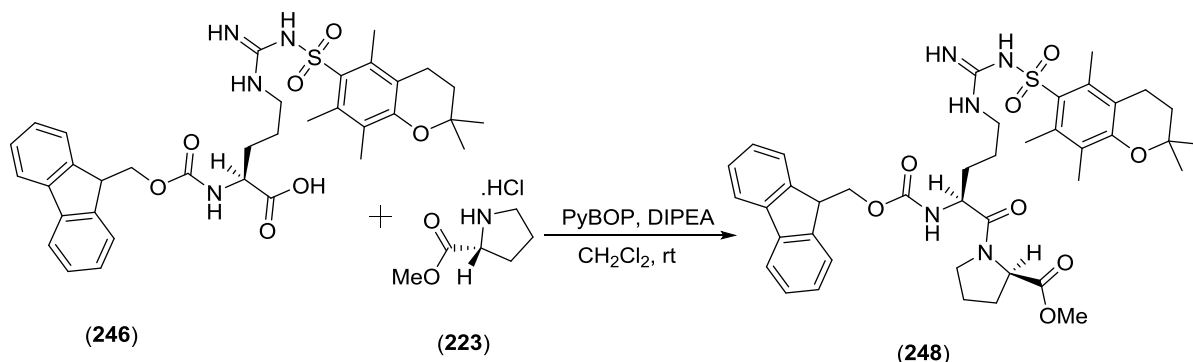
Compound (**244**) (557 mg, 1.50 mmol), HOBt (596 mg, 2.10 mmol) and EDC.HCl (431 mg, 1.50 mmol) were dissolved in anhydrous CH<sub>2</sub>Cl<sub>2</sub> (3.5 mL) and DMF (3.5 mL). The resulting solution was allowed to stir for 30 min at 0 °C. Compound (**223**) (373 mg, 1.50 mmol) and DIPEA (0.52 mL, 3.0 mmol) were added to the resulting mixture which was allowed to warm gradually to room temperature overnight. The reaction mixture was concentrated under vacuum and the residue dissolved in EtOAc (20 mL) and extracted with 5% aq. citric acid (2 x 20 mL), sat. aq. NaHCO<sub>3</sub> (2 x 20 mL) and brine (2 x 20 mL). The organic extract was dried, filtered and the solvent was evaporated to give an oil. The crude material was purified by column chromatography (0-1% MeOH/CH<sub>2</sub>Cl<sub>2</sub> eluent) to give (**245**) as an oil (414 mg, 57%); **IR**  $\nu_{\text{max}}$  (KBr disc) 3296, 3242 (NH), 2933, (CH), 1667 (C=O), 1511, 1441 (Ar, NH), 1240 (CO), 755 (CH Ar); **<sup>1</sup>H NMR** (270 MHz, CDCl<sub>3</sub>)  $\delta$  two rotamers 1.32 (7.1H, s, C(CH<sub>3</sub>)<sub>3</sub>), 1.39 (1.9H, s, C(CH<sub>3</sub>)<sub>3</sub>), 1.71-1.90 (3H, m, 2 x CH $\gamma$  Pro, 1 x H $\beta$  Pro), 2.05-2.09 (1H, m, 1 x H $\beta$  Pro), 2.73-3.01 (2H, m, 2 x H $\beta$  Tyr), 3.07-3.27 (1H, m, 1 x H $\delta$ Pro), 3.50-3.54 (1H, m, 1 x H $\delta$  Pro), 3.62 (2.3H, s, CO<sub>2</sub>CH<sub>3</sub> major rotamer), 3.65 (0.7H, s, CO<sub>2</sub>CH<sub>3</sub> minor rotamer), 4.29-4.38 (0.2H, m, 0.2 x H $\alpha$  Pro minor rotamer), 4.41-4.48 (0.8H, m, H $\alpha$  Pro major rotamer), 4.51-4.60 (1H, m, H $\alpha$ Tyr), 4.78 (2H, s, PhCH<sub>2</sub>-O-), 6.77-6.90 (2H, m Ar-H), 7.01-7.18 (2H, m, Ar-H), 7.29-7.40 (5H, m, Ph).

### Fmoc-L-Arg(Pmc)-L-Pro-OMe (**247**)



Compound (**246**) (994 mg, 1.50 mmol), (**226**) (249 mg, 1.50 mmol) and PyBOP (780 mg, 1.50 mmol) were dissolved in anhydrous CH<sub>2</sub>Cl<sub>2</sub> (7 mL). The reaction mixture was treated with DIPEA (0.52 mL, 3.0 mmol) and then stirred at room temperature overnight until the Fmoc-L-Arg(Pmc)-OH was consumed, as shown by TLC (5% MeOH/CH<sub>2</sub>Cl<sub>2</sub>). The solvent was evaporated and the residue dissolved in EtOAc (20 mL) and extracted with 5% aq. citric acid (3 x 50 mL), sat. aq. NaHCO<sub>3</sub> (3 x 50 mL) and brine (3 x 50 mL). The organic extract was dried, filtered and the solvent evaporated to give a white solid. The crude material was purified by column chromatography (0-1% MeOH/CH<sub>2</sub>Cl<sub>2</sub> eluent) to give (**247**) as a white solid (815 mg, 70%); <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz) δ 1.25 (6H, s, 2 x CH<sub>3</sub> Pmc), 1.58-1.68 (2H, m, 2 x H<sub>γ</sub> Arg), 1.74-1.79 (2H, m, CH<sub>2</sub> Pmc), 1.90-2.03 (5H, m, 2 x H<sub>β</sub> Arg, 1 x H<sub>β</sub>-Pro, 2 x H<sub>γ</sub> Pro), 2.09 (3H, s, Pmc Ar-Me), 2.16-2.21 (1H, m, 1 x H<sub>β</sub> Pro), 2.55-2.62 (6H, m, Pmc 2 x Ar-Me), 3.21-3.22 (2H, m, 2 x H<sub>γ</sub> Arg), 3.56-3.58 (2H, m, 2 x H<sub>δ</sub> Pro), 3.66 (3H, s, CO<sub>2</sub>Me), 4.13-4.17 (1H, m, Fmoc-CH), 4.28-4.35 (2H, m, Fmoc-CH<sub>2</sub>), 4.48-4.51 (2H, m, H<sub>α</sub> Arg, H<sub>α</sub> Pro), 5.97-6.10 (4H, m, 4 x Arg NH), 7.24-7.29 (2H, m, Ar-H Fmoc), 7.34-7.39 (2H, m, Ar-H Fmoc), 7.54-7.56 (2H, m, Ar-H Fmoc), 7.72-7.74 (2H, m, Ar-H Fmoc); <sup>13</sup>C NMR (CDCl<sub>3</sub>, 400 MHz) δ 12.02 (CH<sub>3</sub> Pmc), 17.38 (CH<sub>3</sub> Pmc), 18.41 (CH<sub>3</sub> Pmc), 21.37 (CH<sub>2</sub>, Pmc), 25.01 (2 x CH<sub>2</sub>, CH<sub>2</sub>γ, Arg/Pro), 26.69 (2 x CH<sub>3</sub>, Pmc), 28.93 (2 x CH<sub>2</sub>, CH<sub>2</sub>β, Arg/Pro), 32.82 (CH<sub>2</sub>, Pmc), 40.69 (CH<sub>2</sub>δ Arg), 47.11 (CH, Fmoc), 51.69 (CH<sub>2</sub>δ, Pro), 52.55 (OMe), 58.91 (CH<sub>α</sub>, Arg), 67.01 (CH<sub>α</sub>, Pro), 73.49 (CH<sub>2</sub> Fmoc), 117.80 (SO<sub>2</sub>-CAr Pmc), 119.95, 123.87, 124.96, 127.70 (CH, Ar), 133.54, 134.89, 135.53, 141.27 (C quaternary from Pmc), 143.58, 143.70 (C, quaternary from Fmoc), 153.45 (C=N), 156.00 (C=O).

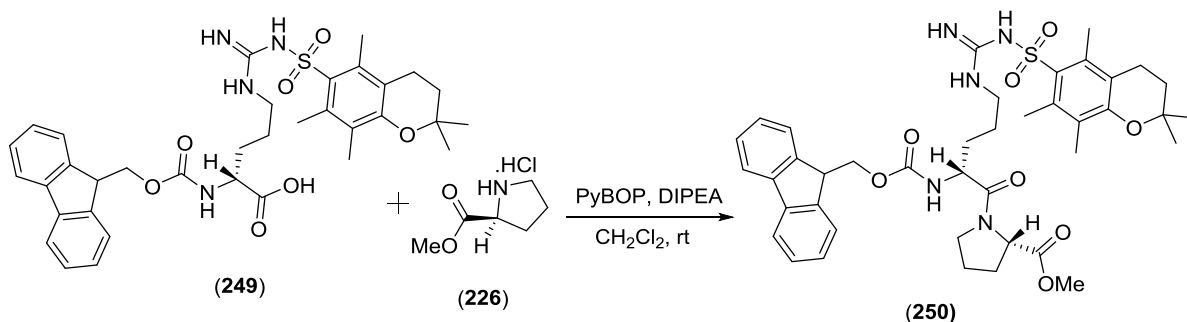
## Fmoc-L-Arg(Pmc)-D-Pro-OMe (**248**)



Compound (**246**) (994 mg, 1.50 mmol), (**223**) (249 mg, 1.5 mmol) and PyBOP (780 mg, 1.5 mmol) were dissolved in anhydrous CH<sub>2</sub>Cl<sub>2</sub> (7 mL). The reaction mixture was treated with DIPEA (0.52 mL, 3 mmol) and then stirred at room temperature overnight until the Fmoc-L-Arg(Pmc)-OH was consumed, as shown by TLC (5% MeOH/CH<sub>2</sub>Cl<sub>2</sub>). The solvent was evaporated and the residue dissolved in EtOAc (20 mL) and extracted with 5% aq. solution of citric acid (3 x 50 mL), sat. aq. solution of NaHCO<sub>3</sub> (3 x 50 mL) and brine (3 x 50 mL). The organic extract was dried and filtered and evaporated to give white solid. The crude material was purified by column chromatography (0-2% MeOH/CH<sub>2</sub>Cl<sub>2</sub> gradient) to give (**248**) as a white solid (985 mg, 85%); [ $\alpha$ ]<sup>21</sup><sub>D</sub> = +1.44 (c 1.04, in MeOH); **Mp** 115-120°C; **IR**  $\nu_{\text{max}}$  (KBr disc) 3438, 3347 (NH), 2951, (CH), 1727, 1632 (C=O), 1549, 1446 (Ar, NH), 1247 (CO), 1109 (SO<sub>2</sub>), 745 (CH Ar); **<sup>1</sup>H NMR** (400 MHz, CDCl<sub>3</sub>) two rotamers  $\delta$  1.25 (6H, s, 2 x CH<sub>3</sub> Pmc), 1.56-1.65 (2H, m, 2 x H $\gamma$ Arg), 1.70-1.78 (2H, m, CH<sub>2</sub> Pmc), 1.80-2.04 (5H, m, 2 x H $\beta$  Arg, 1 x H $\beta$  Pro, 2 x H $\gamma$ -Pro), 2.07 (3H, s, Pmc Ar-Me), 2.12-2.18 (1H, m, 1 x H $\beta$  Pro), 2.51-2.59 (6H, m, 2 x Pmc Ar-Me), 3.10-3.18 (2H, m, 2 x H $\gamma$  Arg), 3.48-3.58 (2H, m, 2 x H $\delta$  Pro), 3.67 (2H, s, CO<sub>2</sub>Me), 3.71 (1H, s, CO<sub>2</sub>Me), 4.10-4.16 (1H, m, Fmoc-CH), 4.26-4.34 (2H, m, Fmoc-CH<sub>2</sub>), 4.37-4.42 (1H, m, H $\alpha$  Arg), 4.46-4.49 (1H, m, H $\alpha$  Pro), 5.97-6.09 (4H, m, 4 x NH, Arg), 7.22-7.28 (2H, m, Ar-H Fmoc), 7.34-7.37 (2H, m, Ar-H Fmoc), 7.51-7.56 (2H, m, Ar-H Fmoc), 7.71-7.73 (2H, m, Ar-H Fmoc); **<sup>13</sup>C NMR** (CDCl<sub>3</sub>, 400 MHz)  $\delta$  12.02 (CH<sub>3</sub> Pmc), 17.36 (CH<sub>3</sub> Pmc), 18.43 (CH<sub>3</sub> Pmc), 21.33 (CH<sub>2</sub>, Pmc), 24.55 (2 x CH<sub>2</sub>, CH<sub>2</sub> $\gamma$  Arg/Pro), 26.67 (2 x CH<sub>3</sub>, Pmc), 28.91 (2 x CH<sub>2</sub>, CH<sub>2</sub> $\beta$  Arg/Pro), 32.74 (CH<sub>2</sub>, Pmc), 40.80 (CH<sub>2</sub> $\delta$  Arg), 47.05 (CH, Fmoc), 52.28 (CH<sub>2</sub> $\delta$  Pro), 52.83 (OCH<sub>3</sub>), 59.21 (CH $\alpha$  Arg), 67.09 (CH $\alpha$  Pro), 73.54 (CH<sub>2</sub>, Fmoc), 117.87 (SO<sub>2</sub>-C Ar Pmc), 119.85, 119.87, 125.09, 127.02, 127.66 (CH Ar), 133.32, 134.79, 135.41, 141.18 (C quaternary from Pmc), 143.56, 143.77 (C quaternary from Fmoc), 153.52

(C=N), 156.05, 156.46, 170.46, 172.43 (C=O); [found (ES+) 774.3570  $[M+H]^+$ ,  $C_{41}H_{52}N_5O_8$  requires 774.3536].

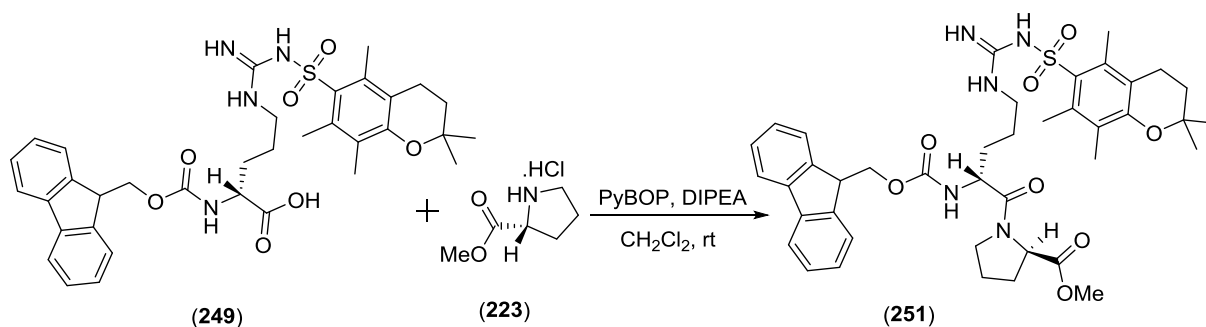
### Fmoc-D-Arg(Pmc)-L-Pro-OMe (**250**)



Fmoc-D-Arg(Pmc)-OH (**249**) (994 mg, 1.50 mmol), (**226**) (249 mg, 1.50 mmol) and PyBOP (780 mg, 1.50 mmol) were dissolved in anhydrous  $CH_2Cl_2$  (7 mL). The reaction mixture was treated with DIPEA (0.52 mL, 3.0 mmol) and the reaction mixture was stirred at room temperature overnight until Fmoc-D-Arg(Pmc)-OH was consumed, as shown by TLC (5% MeOH/ $CH_2Cl_2$ ). The solvent was evaporated and the residue dissolved in EtOAc (20 mL) and extracted with 5% aq. citric acid (3 x 50 mL), sat. aq.  $NaHCO_3$  (3 x 50 mL) and brine (3 x 50 mL). The organic extract was dried, filtered and the solvent evaporated to give a white solid. The crude material was purified by column chromatography (0-2% MeOH/ $CH_2Cl_2$  eluent) to give (**250**) as a white solid (985 mg, 85%);  $[\alpha]^{21}_D = -1.41$  (c 1.56, in MeOH); **Mp** 105-110°C; **IR**  $\nu_{max}$  (KBr disc) 3437, 3341 (NH), 2956, (CH), 1727, 1629 (C=O), 1548, 1446 (Ar, NH), 1244 (CO), 1108 ( $SO_2$ ), 751 (CH Ar);  **$^1H$  NMR** ( $CDCl_3$ , 400 MHz) two rotamers  $\delta$  1.26 (6H, s, 2 x  $CH_3$  Pmc), 1.56-1.68 (2H, m, 2 x  $H_\gamma$  Arg), 1.75-1.82 (2H, m,  $CH_2$  Pmc), 1.89-2.05 (5H, m, 2 x  $H_\beta$  Arg, 1 x  $H_\beta$  Pro, 2 x  $H_\gamma$  Pro), 2.09 (3H, s, Me-Ar, Pmc), 2.14-2.19 (1H, m, 1 x  $H_\beta$  Pro), 2.55-2.61 (6H, m, 2 x Me-Ar, Pmc), 3.10-3.18 (2H, m, 2 x  $H_\gamma$  Arg), 3.51-3.55 (2H, m, 2 x  $H_\delta$  Pro), 3.67 (2H, s,  $CO_2Me$ ), 3.72 (1H, s,  $CO_2Me$ ), 4.13-4.19 (1H, m, CH-Fmoc), 4.31-4.37 (2H, m,  $CH_2$ -Fmoc), 4.40-4.42 (1H, m,  $H_\alpha$  Arg), 4.48-4.52 (1H, m,  $H_\alpha$  Pro), 5.85-5.87 (1H, m, NH Arg), 6.04-6.07 (3H, m, 3 x NH, Arg), 7.25-7.29 (2H, m, Ar-H Fmoc), 7.35-7.39 (2H, m, Ar-H Fmoc), 7.51-7.57 (2H, m, Ar-H Fmoc), 7.72-7.74 (2H, m, Ar-H Fmoc);  **$^{13}C$  NMR** ( $CDCl_3$ , 400 MHz)  $\delta$  12.02 ( $CH_3$  Pmc), 17.36 ( $CH_3$  Pmc), 18.43 ( $CH_3$  Pmc), 21.35 ( $CH_2$ , Pmc), 24.54 ( $CH_2$ ,  $CH_2\gamma$  Arg/Pro), 26.69  $CH_3$  (2 x  $CH_3$ , Pmc) 28.96 ( $CH_2$ ,  $CH_2\beta$  Arg/Pro), 32.76 ( $CH_2$ , Pmc), 40.92 ( $CH_2\delta$  Arg), 47.10 (CH, Fmoc), 52.22 ( $CH_2\delta$  Pro), 55.36 ( $OCH_3$ ), 59.18 ( $CH_\alpha$

Arg), 67.06 (CH $\alpha$  Pro), 73.54 (CH $_2$  Fmoc), 117.87 (C, C-SO $_2$ , Ar Pmc), 119.89, 123.96, 125.05, 127.03, 127.68 (CH Ar), 133.38, 134.78, 135.41 C (C quaternary from Pmc), 141.22, 143.75 C (C quaternary from Fmoc), 153.52 (C=N), 155.93, 156.44, 172.27 (C=O); [found (ES $^+$ ) 796.3379 [M+Na] $^+$ , requires 796.3356 C $_{41}$ H $_{51}$ N $_5$ O $_8$ SNa].

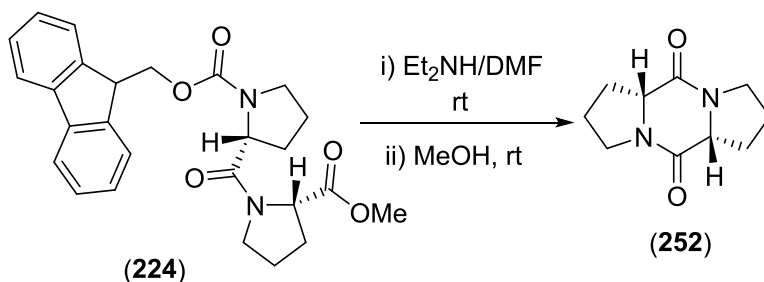
### Fmoc-D-Arg(Pmc)-D-Pro-OMe (**251**)



Compound (**249**) (407 mg, 0.61 mmol), (**223**) (101 mg, 0.61 mmol) and PyBOP (317 mg, 0.61 mmol) were dissolved in anhydrous CH $_2$ Cl $_2$  (7 mL). The reaction mixture was treated with DIPEA (0.21 mL, 1.2 mmol) and then stirred at room temperature overnight until the Fmoc-D-Arg(Pmc)-OH was consumed, as shown by TLC (5% MeOH/CH $_2$ Cl $_2$ ). The solvent was evaporated and the residue dissolved in EtOAc (20 mL) and extracted with 5% aq. citric acid (3 x 50 mL), sat. aq. NaHCO $_3$  (3 x 50 mL) and brine (3 x 50 mL). The organic extract was dried and filtered to give a white solid. The crude material was purified by column chromatography (0-2% MeOH/CH $_2$ Cl $_2$  eluent) to give (**251**) as a white solid (357 mg, 76%); **Mp** 117-122  $^{\circ}$ C; **IR**  $\nu_{\text{max}}$  (KBr disc) 3438, 3341 (NH), 2951, (CH), 1727, 1634 (C=O), 1549, 1446 (Ar, NH), 1248 (CO), 1109 (SO $_2$ ), 742 (CH Ar);  **$^1\text{H}$  NMR** (CDCl $_3$ , 400 MHz)  $\delta$  1.26 (6H, s, 2 x CH $_3$  Pmc), 1.58-1.70 (2H, m, 2 x H $\gamma$ -Arg), 1.77 (2H, t,  $J$  8 Hz, CH $_2$  Pmc), 1.89-2.05 (5H, m, 2 x H $\beta$  Arg, 2 x H $\gamma$  Pro, 1 x H $\beta$  Pro), 2.10 (3H, s, Me-Ar, Pmc), 2.16-2.24 (1H, m, 1 x H $\beta$  Pro), 2.56-2.63 (6H, m, 2 x Me-Ar, Pmc), 3.21-3.23 (2H, m, 2 x H $\gamma$  Arg), 3.54-3.60 (2H, m, 2 x H $\delta$  Pro), 3.65 (3H, s, CO $_2$ Me), 4.14-4.17 (1H, m, CH-Fmoc), 4.29-4.38 (2H, m, CH $_2$ -Fmoc), 4.48-4.51 (2H, m, H $\alpha$  Arg, H $\alpha$  Pro), 5.84 (1H, m, 1 x NH Arg), 5.94-5.96 (1H, m, 1 x NH Arg), 6.08 (2H, m, 2 x NH Arg), 7.25-7.29 (2H, m, Ar-H Fmoc), 7.35-7.39 (2H, m, Ar-H Fmoc), 7.51-7.56 (2H, m, Ar-H Fmoc), 7.72-7.74 (2H, m, Ar-H Fmoc);  **$^{13}\text{C}$  NMR** (CDCl $_3$ , 400 MHz)  $\delta$  12.03 (CH $_3$  Pmc), 17.39 (CH $_3$  Pmc), 18.43 (CH $_3$  Pmc), 21.36 (CH $_2$ , Pmc), 23.78 (CH $_2\gamma$  Arg), 24.98 (CH $_2\gamma$  Pro) 26.69 (2 x CH $_3$ , Pmc), 28.92 (CH $_2\beta$  Arg), 29.05 (CH $_2\beta$  Pro) 32.80 (CH $_2$ , Pmc), 40.68 (CH $_2\delta$  Arg), 47.09 (CH, Fmoc), 51.83 (CH $_2\delta$  Pro), 52.48 (OCH $_3$ ), 58.96 (CH $\alpha$

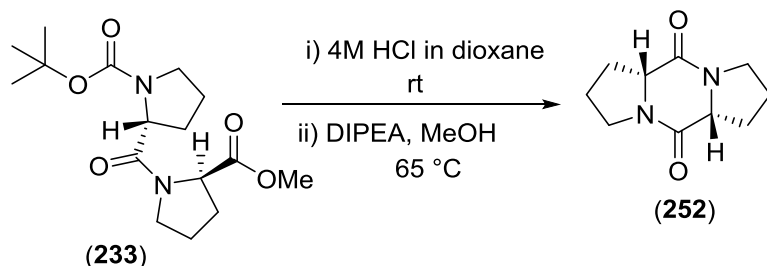
Arg), 67.00 (CH $\alpha$  Pro), 73.50 (CH<sub>2</sub>, Fmoc), 117.81 (CAr-SO<sub>2</sub> Pmc), 119.88, 119.91, 123.87, 125.03, 125.08, 126.99, 127.64 (CH, Ar), 133.55, 134.85, 135.49 (C quaternary from Pmc), 141.21, 143.64, 143.72 (C quaternary from Fmoc), 153.44 (C=N), 156.44, 170.46, 172.43 (C=O ester); [found (ESI+) 774.3550 [M+H]<sup>+</sup>, C<sub>41</sub>H<sub>52</sub>N<sub>5</sub>O<sub>8</sub> requires 774.3536].

#### Attempted preparation of cyclo(D-Pro-D-Pro) (**252**)



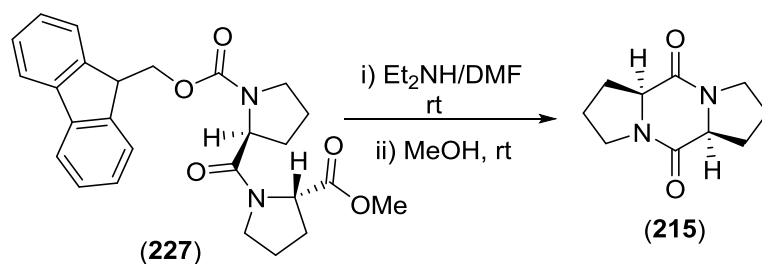
Compound (**224**) (149 mg, 0.33 mmol) was dissolved in anhydrous DMF (1.5 mL) and treated with Et<sub>2</sub>NH (1.5 mL, 0.14 mol), at room temperature for 10 min, until the starting material was consumed as shown by TLC (EtOAc/petroleum ether: 1/1). The reaction mixture was then co-evaporated to dryness with toluene to remove residual DMF to give a brown oil. The residue was dissolved in MeOH (15 mL) and was stirred at 65 °C, for 2 h, until the lower UV-active spot was replaced by a new spot at **R<sub>f</sub>** 0.42 (50% EtOAc/petroleum ether). The reaction mixture was concentrated under vacuum and triturated with petroleum ether. The compound was purified by column chromatography using MeOH/CH<sub>2</sub>Cl<sub>2</sub> as eluent; this gave the cyclic dipeptide (**252**), plus the by-product from Fmoc deprotection.

### Cyclo(D-Pro-D-Pro) (**252**)<sup>222</sup>



Compound (**233**) (230 mg, 0.71 mmol) was treated with 4M HCl-dioxane (2 mL) at room temperature for 30 min, until the starting material was consumed as shown by TLC. The reaction mixture was evaporated to dryness and the residue redissolved in anhydrous MeOH (10 mL) and DIPEA (0.50 mL, 2.9 mmol) and the reaction mixture was stirred at 65 °C overnight. The solvent were evaporated and the residue was dissolved in H<sub>2</sub>O and extracted with CH<sub>2</sub>Cl<sub>2</sub> (2 x 10). The combined organic layers were filtered and dried to give (**252**) as a white solid (45 mg, 33%) (lit<sup>259</sup> Mp 141-143 °C); <sup>1</sup>H NMR (CD<sub>3</sub>OD, 400 MHz) δ 1.19-2.22 (6H, m, 4 x H<sub>γ</sub> Pro, 2 x H<sub>β</sub> Pro), 2.32-2.39 (2H, m, 2 x H<sub>β</sub> Pro), 3.55-3.59 (4H, m, 4 x H<sub>δ</sub> Pro), 4.41 (2H, t, *J* 8 Hz, 2 x H<sub>α</sub> Pro).<sup>222</sup>

### Attempted preparation of cyclo(L-Pro-L-Pro) (**215**)

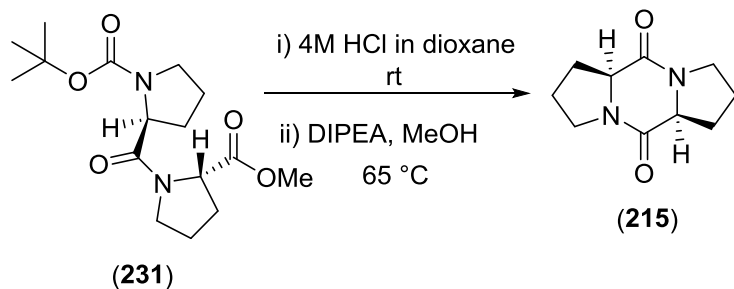


Compound (**227**) (342 mg, 0.76 mmol) was dissolved in anhydrous DMF (1.5 mL) and treated with Et<sub>2</sub>NH (1.5 mL, 0.14 mol), at room temperature for 10 min, until disappearance of the starting material was shown by TLC (10% MeOH/CH<sub>2</sub>Cl<sub>2</sub>). The reaction mixture was evaporated to dryness and then co-evaporated with toluene to remove residual DMF to give an oil. The residue was dissolved in MeOH (15 mL) and was then stirred at 65 °C, for 2 h, until the lower UV-active was replaced by a new spot at *R<sub>f</sub>* 0.86 (10% MeOH/CH<sub>2</sub>Cl<sub>2</sub>). The reaction mixture was concentrated under vacuum and triturated with petroleum ether. The compound



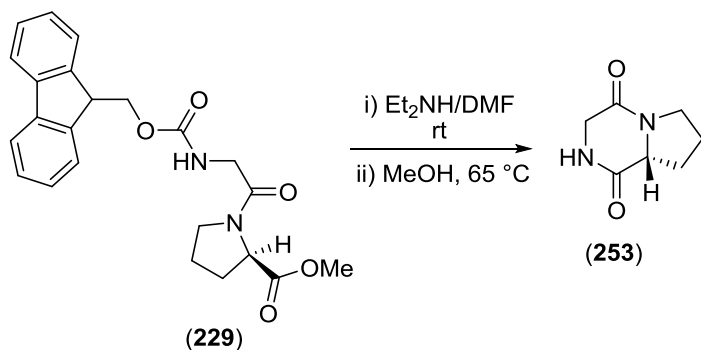
was purified by column chromatography using MeOH/CH<sub>2</sub>Cl<sub>2</sub> as eluent. This gave the cyclic dipeptide (**215**), plus the by-product from Fmoc deprotection.

**Cyclo(L-Pro-L-Pro) (**215**)**<sup>258</sup>



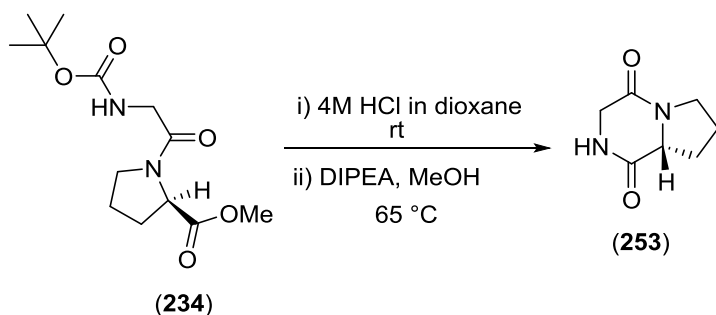
Compound (**231**) (150 mg, 0.46 mmol) was treated with 4M HCl-dioxane (2 mL) at room temperature for 40 min, until the starting material was consumed as shown by TLC. The reaction mixture was evaporated to dryness and the residue redissolved in anhydrous MeOH (10 mL) and DIPEA (0.40 mL, 2.3 mmol) and the reaction mixture was stirred at 65 °C overnight. The solvent were evaporated and the residue was dissolved in H<sub>2</sub>O and extracted with CH<sub>2</sub>Cl<sub>2</sub> (2 x 10 mL). The combined organic layers were filtered and dried to give (**215**) as a white solid (29.8 mg, 33%); **Mp** 138-142 °C, (lit<sup>259</sup> 141-143 °C); **IR**  $\nu_{\text{max}}$  (KBr disc) 3435, 3209 (NH), 2957 (CH), 1661 (C=O), 1458 (NH); **<sup>1</sup>H NMR** (CD<sub>3</sub>OD, 400 MHz)  $\delta$  1.97-2.19 (6H, m, 4 x H $\gamma$  Pro, 2 x H $\beta$  Pro), 2.32-2.40 (2H, m, 2 x H $\beta$  Pro), 3.51-3.62 (4H, m, 4 x H $\delta$  Pro), 4.42 (2H, t, *J* 8 Hz, 2 x H $\alpha$  Pro); **<sup>13</sup>C NMR** (CDCl<sub>3</sub>, 101 MHz)  $\delta$  24.19 CH<sub>2</sub> $\gamma$  Pro, 28.74 CH<sub>2</sub> CH<sub>2</sub> $\beta$  Pro, 46.22 CH<sub>2</sub> $\delta$  Pro, 61.76 CH $\alpha$  Pro, 168.63 (C=O); [found (ESI+) 195.1111 [M+H]<sup>+</sup>, C<sub>10</sub>H<sub>15</sub>N<sub>2</sub>O<sub>2</sub> requires 195.1133].

### Attempted preparation of cyclo (Gly-D-Pro) (**253**)



Compound (**229**) (347 mg, 0.850 mmol) was dissolved in anhydrous DMF (15 mL) and treated with  $\text{Et}_2\text{NH}$  (1.5 mL, 0.14 mol), at room temperature for 10 min, until the starting material was consumed as shown by TLC (10%  $\text{MeOH}/\text{CH}_2\text{Cl}_2$ ) and a new lower UV-active spot appeared. The reaction mixture was evaporated to dryness and then co-evaporated with toluene to remove residual DMF to give a yellow oil. The residue was dissolved in  $\text{MeOH}$  (15 mL) and stirred at  $65\text{ }^\circ\text{C}$ , for 24 h, until the lower UV-active spot was replaced by a new spot at  $R_f$  0.42 (10%  $\text{MeOH}/\text{CH}_2\text{Cl}_2$ ). The reaction mixture was concentrated under vacuum and triturated with petroleum ether. The compound was purified by column chromatography using  $\text{MeOH}/\text{CH}_2\text{Cl}_2$  as eluent. This gave the cyclic dipeptide (**253**), plus the by-product from Fmoc deprotection.

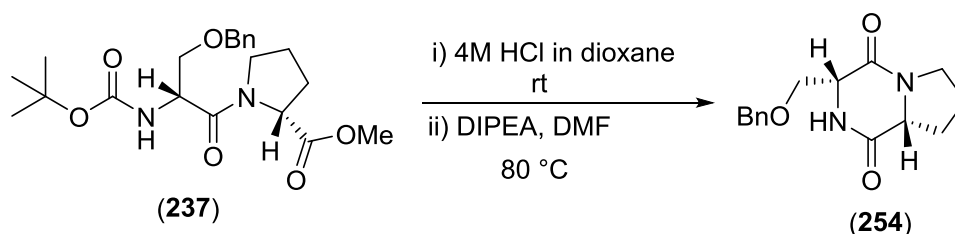
### Cyclo (Gly-D-Pro) (**253**)<sup>265</sup>



Compound (**234**) (157 mg, 0.55 mmol) was treated with 4M  $\text{HCl}$ -dioxane (2 mL) at room temperature for 45 min, until the starting material was consumed as shown by TLC at  $R_f$  0.73 (5%  $\text{MeOH}/\text{CH}_2\text{Cl}_2$ ). The reaction mixture was evaporated to dryness and the residue redissolved in anhydrous  $\text{MeOH}$  (10 mL) and  $\text{DIPEA}$  (0.40 mL, 2.3 mmol) and the reaction

mixture was stirred at 65 °C overnight. The solvent was evaporated and the residue was dissolved in H<sub>2</sub>O and extracted with CH<sub>2</sub>Cl<sub>2</sub> (2 x 10 mL). The combined organic layers were filtered and dried to give a white solid (155 mg). The crude material was purified by column chromatography using 10-20% EtOAc/petroleum ether as eluent, to give (**253**) as a white solid (37.8 mg, 45%); **Mp** 201-205 °C (lit<sup>264</sup> **Mp** 205-208 °C); **IR**  $\nu_{\text{max}}$  (KBr disc) 3414 (NH), 2922, 2852 (CH), 1655 (C=O), 1458 (NH); **<sup>1</sup>H NMR** (CD<sub>3</sub>OD, 400 MHz)  $\delta$  1.97 (3H, m, 2 x H $\gamma$  Pro, 1 x H $\beta$  Pro), 2.36-2.42 (1H, m, 1 x H $\beta$  Pro), 3.56-3.65 (2H, m, 2 x H $\delta$  Pro), 3.81 (1H, d, *J* 16.0 Hz, CH $\alpha$  Gly), 4.15- 4.19 (1H, m, 1 x H $\alpha$ Gly), 4.28-4.31 (1H, m, H $\alpha$  Pro); **<sup>13</sup>C NMR** (CDCl<sub>3</sub>, 101 MHz)  $\delta$  23.33 (CH<sub>2</sub> $\gamma$  Pro), 29.40 (CH<sub>2</sub> $\beta$  Pro), 46.34 (CH<sub>2</sub> $\delta$  Pro), 47.03 (CH<sub>2</sub> $\alpha$  Gly), 59.88 (CH $\alpha$  Pro), 166.48, 172.01 (C=O); [found (ESI+) 155.0815 [M+H]<sup>+</sup>, C<sub>7</sub>H<sub>11</sub>N<sub>2</sub>O<sub>2</sub> requires 155.0820].

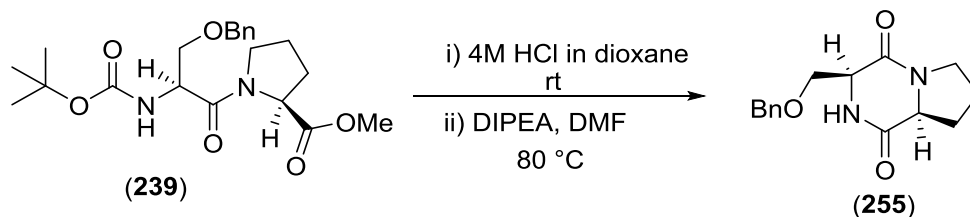
#### Cyclo(D-Ser(OBn)-D-Pro) (**254**)



Compound (**237**) (304 mg, 0.75 mmol) was treated with 4M HCl-dioxane at room temperature for 40 min, until the starting material was consumed as shown by TLC at **R<sub>f</sub>** 0.77 (10% MeOH/CH<sub>2</sub>Cl<sub>2</sub>). The reaction mixture was evaporated to dryness and the residue redissolved in DMF (7 mL) and DIPEA (0.60 mL, 3.44 mmol) and the reaction mixture was stirred at 80 °C, overnight. The reaction mixture was evaporated to dryness and then co-evaporated with toluene to remove residual DMF to give a brown oil. The resulting brown oil was purified by column chromatography (0-2% MeOH/CH<sub>2</sub>Cl<sub>2</sub> eluent) and the oil obtained was dissolved in CH<sub>2</sub>Cl<sub>2</sub> and precipitated with petroleum ether to give (**254**) as a brown solid (94 mg, 46%); **RP-HPLC** (analytical system, gradient 1) **R<sub>t</sub>** = 6.68 min; **Mp** 133-135 °C; **IR**  $\nu_{\text{max}}$  (KBr disc) 3443, 3237 (NH), 2929, 2883 (CH), 1677 (C=O), 1448 (Ar, NH), 1109 (CO), 741 (CH Ar); **<sup>1</sup>H NMR** (CDCl<sub>3</sub>, 270 MHz)  $\delta$  1.90-2.07 (3H, m, 1 x H $\gamma$  Pro, 2 x H $\gamma$  Pro), 2.32-2.38 (1H, m, 1 x H $\beta$  Pro), 3.51-3.64 (3H, m, 1 x H $\beta$ -Ser, 2 x H $\delta$  Pro), 4.05-4.10 (2H, m, 1 x H $\beta$  Ser, H $\alpha$  Ser), 4.31 (1H, dd, *J* 10.3, 3.8 Hz H $\alpha$  Pro), 4.55 (2H, ABq, *J* 6.1, 1.6 Hz, CH<sub>2</sub>Ph), 6.25 (1H, br, NH), 7.25-

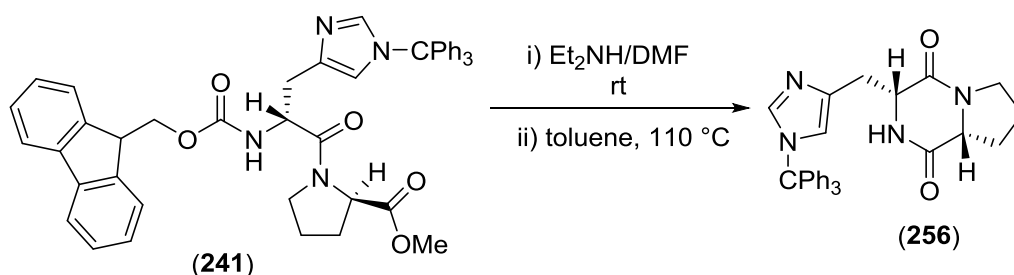
7.35 (5H, m, Ph);  $^{13}\text{C}$  NMR (CDCl<sub>3</sub>, 101 MHz)  $\delta$  22.48 (CH<sub>2</sub> $\gamma$  Pro), 28.38 (CH<sub>2</sub> $\beta$  Pro), 45.30 (CH<sub>2</sub> $\delta$  Pro), 54.44 (CH $\alpha$  Ser), 58.93 (CH $\alpha$  Pro), 68.77 (CH<sub>2</sub> $\beta$  Ser), 73.61 (CH<sub>2</sub>Ph), 127.93, 128.14 128.60, 135.05 (CH Ar, C quaternary), 163.49, 168.88 (C=O).

### Cyclo(L-Ser(OBn)-L-Pro (255)



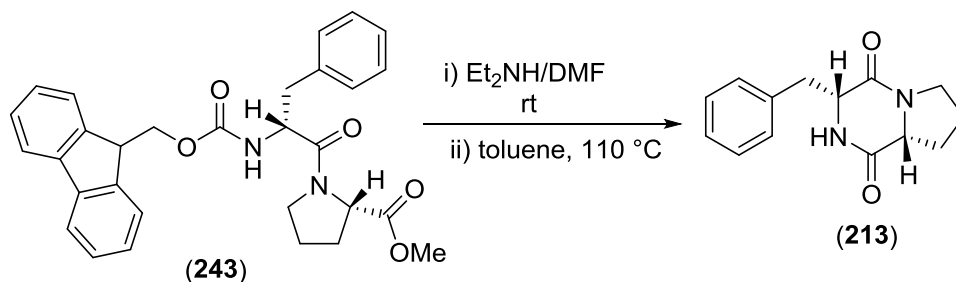
Compound **(239)** (430 mg, 1.06 mmol) was treated with 4M HCl-dioxane (3 mL) at room temperature for 40 min, until the starting material was consumed as shown by TLC at **R<sub>f</sub>** 0.22 (5% MeOH/CH<sub>2</sub>Cl<sub>2</sub>). The reaction mixture was evaporated to dryness and the residue redissolved in DMF (7 mL) and DIPEA (0.60 mL, 3.4 mmol) and the reaction mixture was stirred at 80 °C, overnight. The reaction mixture was evaporated to dryness and then co-evaporated with toluene to remove residual DMF to give brown oil. The resulting brown oil was purified by column chromatography (2% MeOH/CH<sub>2</sub>Cl<sub>2</sub> eluent), to give **(255)** as a pale brown solid (153 mg, 53%);  $[\alpha]^{21}_{\text{D}} = -9.06$  (c 1.38, in MeOH); **Mp** 135-138 °C; **IR**  $\nu_{\text{max}}$  (KBr disc) 3439, 3227 (NH), 2876 (CH), 1676 (C=O), 1448 (Ar, NH), 1110 (CO), 741 (CH Ar);  $^1\text{H}$  NMR (CDCl<sub>3</sub>, 400 MHz)  $\delta$  1.82-1.98 (1H, m, 1 x H $\gamma$ Pro), 1.99-2.12 (2H, m, 1 x H $\gamma$  Pro, 1 x H $\beta$  Pro), 2.30-2.40 (1H, m, 1 x H $\beta$  Pro), 3.44-3.65 (3H, m, 1 x H $\beta$  Ser, 2 x H $\delta$  Pro), 4.07-4.12 (2H, m, 1 x H $\beta$  Ser, H $\alpha$  Ser), 4.30 (1H, dd, *J* 9.4, 3.3 Hz, H $\alpha$  Pro), 4.55 (2H, ABq, *J* 11.6 Hz CH<sub>2</sub>Ph), 6.25 (1H, br, NH), 7.25-7.35 (5H, m, Ph);  $^{13}\text{C}$  NMR (CDCl<sub>3</sub>, 101 MHz)  $\delta$  22.41 (CH<sub>2</sub> $\gamma$  Pro), 28.31 (CH<sub>2</sub> $\beta$  Pro), 45.22 (CH<sub>2</sub> $\delta$  Pro), 54.47 (CH $\alpha$ Ser), 58.87 (CH $\alpha$  Pro), 68.75 (CH<sub>2</sub> $\beta$  Ser), 73.55 (CH<sub>2</sub>Ph), 127.85, 128.03, 128.51, 137.08 (CH Ar, C quaternary), 163.47, 168.90 (C=O).

### Cyclo(D-His(Trt)-D-Pro) (256)



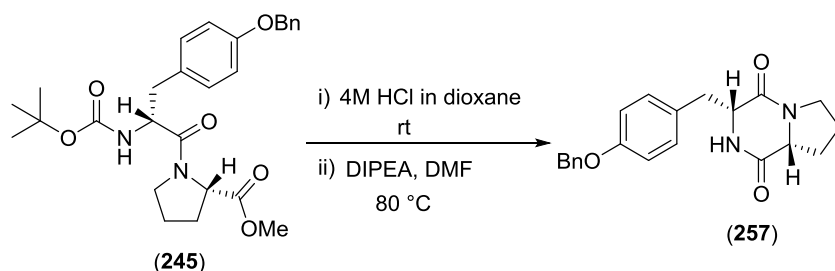
Compound (**241**) (348 mg, 0.48 mmol) was dissolved in anhydrous DMF (1.5 mL) and treated with Et<sub>2</sub>NH (1.5 mL, 0.14 mol), at room temperature for 10 min, until the starting material was consumed as shown by TLC (10% MeOH/CH<sub>2</sub>Cl<sub>2</sub>). The reaction mixture was evaporated to dryness and then co-evaporated with toluene to remove residual DMF to give a colourless oil. The residue was dissolved in toluene (15 mL) and was stirred at 110 °C, for 24 h, until the lower UV-active spot was replaced by a new spot at **R<sub>f</sub>** 0.51 TLC (5% MeOH/CH<sub>2</sub>Cl<sub>2</sub>). The crude material was purified by column chromatography (0-3% MeOH/CH<sub>2</sub>Cl<sub>2</sub> eluent) to give (**256**) as a pale white solid (88 mg, 38%); **RP-HPLC** (analytical system, gradient 1), **R<sub>t</sub>** = 7.2 min; **Mp** above 230 °C; **IR**  $\nu_{\text{max}}$  (KBr disc) 3439, 3236 (NH), 2892 (CH), 1662 (C=O), 1431 (Ar, NH), 756 (CH Ar); **<sup>1</sup>H NMR** (CDCl<sub>3</sub>, 270 MHz)  $\delta$  1.82-2.06 (3H, m, 1 x H $\beta$  Pro, 2 x H $\gamma$  Pro), 2.30-2.35 (1H, m, 1 x H $\beta$  Pro), 2.60-2.79 (1H, dd, *J* 10.7 Hz, 4.4 Hz, 1 x H $\beta$  His), 3.34 (1H, dd, *J* 12.1 Hz, 3.0 Hz, 1 x H $\beta$  His), 3.51-3.55 (2H, m, 2 x H $\delta$  Pro), 4.04 (1H, t, *J* 7.4 Hz, H $\alpha$  His), 4.17 (1H, d, *J* 9.4 Hz, H $\alpha$  Pro), 6.66 (1H, br, NH), 6.95-7.42 (16H, 3 x Ph, 1 x H imidazole ring), 7.63 (1H, s, H imidazole ring); **<sup>13</sup>C NMR** (CDCl<sub>3</sub>, 101 MHz)  $\delta$  22.58 (CH<sub>2</sub> $\gamma$  Pro), 28.15 (CH<sub>2</sub> $\beta$  Pro), 28.40 (CH<sub>2</sub> $\beta$  His), 45.40 (CH<sub>2</sub> $\delta$  Pro), 55.79 (CH $\alpha$  His), 59.05 (CH $\alpha$  Pro), 119.23 (CH imidazole ring), 128.07, 129.67 (CH Ar), 137.00 (C quaternary), 138.93 (C=N), 142.20 (C quaternary from CPh<sub>3</sub>), 165.45, 169.28 (C=O); [found (ESI<sup>+</sup>) 477.2281 [M+H]<sup>+</sup>, C<sub>30</sub>H<sub>29</sub>N<sub>4</sub>O<sub>2</sub> requires 477.2285].

### Cyclo(D-Phe-D-Pro) (**213**)<sup>239</sup>



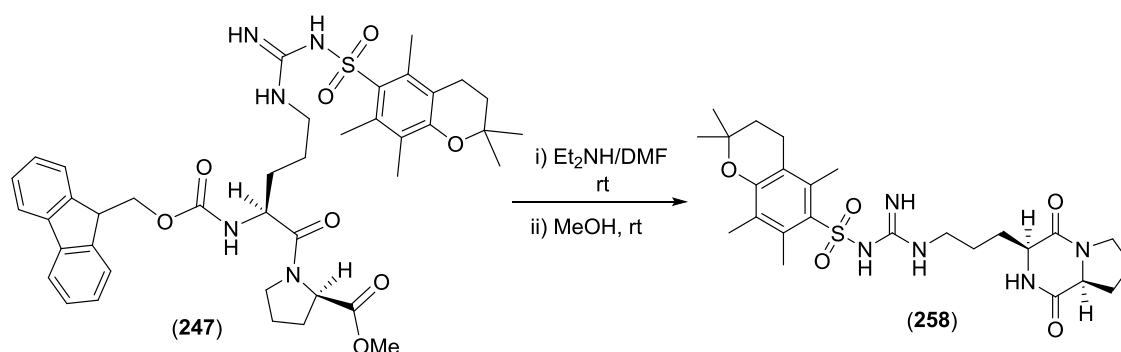
Compound (**243**) (350 mg, 0.70 mmol) was dissolved in anhydrous DMF (3 mL) and treated with Et<sub>2</sub>NH (3.0 mL, 0.28 mol), at room temperature for 15 min, when the starting material was consumed as shown by TLC (10% MeOH/CH<sub>2</sub>Cl<sub>2</sub>). The reaction mixture was evaporated to dryness and then co-evaporated with toluene to remove residual DMF to give an oil. The residue was dissolved in toluene (15 mL) was stirred at 110 °C, for 96 h, until the lower UV-active spot was replaced by a new spot TLC (5% MeOH/CH<sub>2</sub>Cl<sub>2</sub>). The crude material was dissolved in CH<sub>2</sub>Cl<sub>2</sub> and purified by column (2-3% MeOH/CH<sub>2</sub>Cl<sub>2</sub> eluent) to give (**213**) as a brown oil (20 mg, 12%), (lit<sup>260</sup> **Mp** 130-132 °C); **RP-HPLC** (analytical system, gradient 1), *R*<sub>t</sub> = 6.3 min); **IR**  $\nu_{\text{max}}$  (thin film) 3247 (NH), 2995 (CH), 1669 (C=O), 1439 (Ar, NH), 753 (CH Ar); **<sup>1</sup>H NMR** (CDCl<sub>3</sub>, 270 MHz)  $\delta$  1.89-1.98 (3H, m, 1 x H $\beta$  Pro, 2 x H $\gamma$  Pro), 2.28-2.33 (1H, m, 1 x H $\beta$  Pro), 2.77 (1H, dd, *J* 13.9, 10.5 Hz, 1 x H $\beta$  Phe), 3.48-3.64 (3H, m, 1 x H $\beta$ -Phe, 2 x H $\delta$  Pro), 4.04 (1H, t, *J* 7.6 Hz, H $\alpha$  Phe), 4.25 (1H, dd, *J* 10.5, 2.7 Hz, H $\alpha$ Pro), 5.76 (1H, br, NH), 7.21-7.43 (5H, m, Ph); **<sup>13</sup>C NMR** (CDCl<sub>3</sub>, 101 MHz)  $\delta$  22.50 (CH<sub>2</sub> $\gamma$  Pro), 28.31 (CH<sub>2</sub> $\beta$  Pro), 36.75 (CH<sub>2</sub> $\beta$  Phe), 45.40 (CH<sub>2</sub> $\delta$  Pro), 56.16 (CH $\alpha$  Phe), 59.09 (CH $\alpha$  Pro), 127.50, 129.01, 129.10 (CH Ar), 135.90 (C quaternary), 165.03, 169.39 (C=O); [found (ES<sup>+</sup>) 267.1093 [M+Na]<sup>+</sup>, C<sub>14</sub>H<sub>16</sub>N<sub>2</sub>O<sub>2</sub>Na requires 267.1103].

### Cyclo (D-Tyr(OBn)-D-Pro) (257)



Compound (**257**) (414 mg, 0.86 mmol) was treated with 4M HCl-dioxane (2 mL) at room temperature for 40 min, until the starting material was consumed as shown by TLC. The reaction mixture was evaporated to dryness and the residue redissolved in DMF (7 mL) and DIPEA (0.90 mL, 5.2 mmol) and stirred at 80 °C, overnight. The reaction mixture was evaporated to dryness and then co-evaporated with toluene to remove residual DMF to give a brown oil. The resulting brown oil was purified by column chromatography (0-2% MeOH/CH<sub>2</sub>Cl<sub>2</sub> eluent), to give (**257**) as an oil (193 mg, 64%); <sup>1</sup>H NMR (CD<sub>3</sub>OD, 270 MHz) δ 1.67-1.94 (3H, m, 2 x H<sub>γ</sub> Pro, 1 x H βPro), 2.12-2.35 (1H, m, 1 x Hβ Pro), 2.81 (1H, dd, *J* 23.2, 8.5 1 x Hβ Tyr), 3.34-3.64 (3H, m, 1 x Hβ Tyr, 2 x Hδ Pro), 3.88-4.25 (1H, m, H<sub>α</sub> Pro), 4.12-4.24 (1H, m, H<sub>α</sub> Tyr), 4.98 (2H, s, PhCH<sub>2</sub>-O), 6.38 (1H, s, NH), 6.78-6.95 (2H, m, Ar-H), 7.05-7.18 (2H, m, Ar-H), 7.20-7.46 (5H, m, Ph).

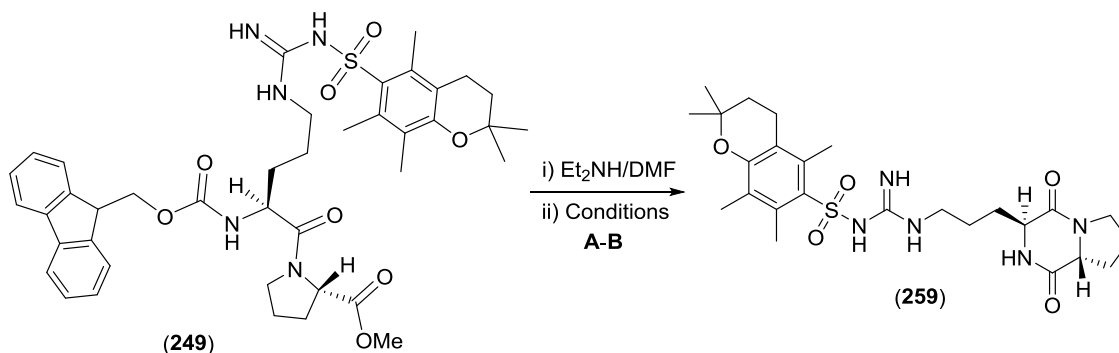
### Cyclo (L-Arg(Pmc)-L-Pro) (258)



Compound (**247**) (808 mg, 1.04 mmol) was dissolved in anhydrous DMF (2 mL) and treated with Et<sub>2</sub>NH (2.0 mL, 0.19 mol), at room temperature for 30 min, until the starting material was consumed as shown by TLC (10% MeOH/CH<sub>2</sub>Cl<sub>2</sub>). The solvent was evaporated to dryness then co-evaporated with toluene to remove residual DMF to give an oil. The residue was dissolved

in MeOH (20 mL) and was stirred at room temperature, for 2 days, until the lower UV-active spot was replaced by a new spot at  $R_f$  0.42 (10% MeOH/CH<sub>2</sub>Cl<sub>2</sub>). The crude material was purified by column chromatography (5-15% MeOH/CH<sub>2</sub>Cl<sub>2</sub> eluent) to give (**258**) as a yellow solid (482 mg, 89%); **RP-HPLC** (analytical system, gradient 2)  $R_t$  = 12.5 min);  $[\alpha]_D^{19}$  = -2.47 (c 3.37, in MeOH); **Mp** 108-112 °C; **IR**  $\nu_{max}$  (KBr disc) 3447, 3349 (NH), 2971, 2935 (CH), 1661, 1551 (C=O), 1441 (Ar, NH), 1298, 1245 (CO), 1110 (SO<sub>2</sub>), 749, 666, 612 (CH Ar); **<sup>1</sup>H NMR** (CDCl<sub>3</sub>, 400 MHz)  $\delta$  1.29 (6H, s, 2 x Me, Pmc), 1.63-1.72 (2H, m, 2 x H $\gamma$  Arg), 1.79 (2H, t,  $J$  6.8 Hz, CH<sub>2</sub>, Pmc) 1.84-2.06 (5H, 2 x H $\gamma$  Pro, 1 x H $\beta$  Pro, 2 x H $\beta$  Arg), 2.09 (3H, s, Me-Ar Pmc), 2.33-2.40 (1H, m, 1 x H $\beta$  Pro), 2.54 (6H, m, 2 x CH<sub>3</sub>, Ar-Me, Pmc), 2.61 (2H, t,  $J$  6.8 Hz, CH<sub>2</sub>, Pmc), 3.17-3.21 (2H, m, 2 x H $\delta$  Arg), 3.47-3.60 (2H, m, 2 x H $\delta$  Pro), 4.06-4.11 (2H, m, H $\alpha$  Arg, H $\alpha$  Pro), 6.23-6.30 (3H, m, 3 x NH Arg); **<sup>13</sup>C NMR** (CDCl<sub>3</sub>, 101 MHz)  $\delta$  12.05, 17.38, 18.43 CH<sub>3</sub> (Pmc, Me), 21.34 (2 x CH<sub>2</sub> Pmc), 22.49 (CH<sub>2</sub> $\gamma$  Pro, CH<sub>2</sub> $\gamma$  Arg), 26.70, 26.72 (Me-Ar, Pmc) 28.19 (CH<sub>2</sub> $\beta$  Pro, CH<sub>2</sub> $\beta$  Arg), 32.73 (CH<sub>2</sub> $\delta$  Arg), 45.23 (CH<sub>2</sub> $\delta$  Pro), 54.86 (CH $\alpha$  Arg), 59.02 (CH $\alpha$  Pro), 73.61 (C quaternary Pmc), 117.95, 123.95, 133.25, 134.75, 135.32 153.51 (C quaternary), 156.40, 165.73, 170.75, 170.79 (2 x C=O, C=N).

#### Cyclo (L-Arg(Pmc)-D-Pro) (**259**)



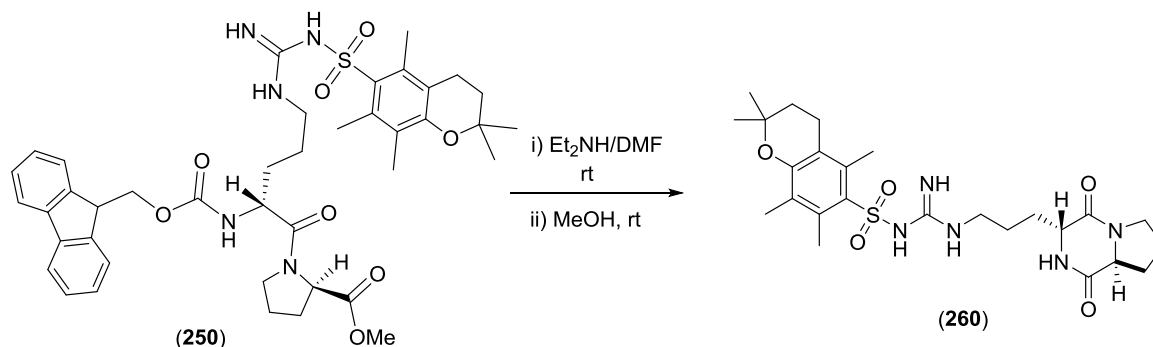
**Method A:** Compound (**249**) (904 mg, 1.17 mmol) was dissolved in anhydrous DMF (2 mL) and treated with Et<sub>2</sub>NH (2 mL, 0.19 mol), at room temperature for 30 min, until the starting material was consumed as shown by TLC (10% MeOH/CH<sub>2</sub>Cl<sub>2</sub>). The reaction mixture was evaporated to dryness then co-evaporated with toluene to remove residual DMF to give an oil. The residue was dissolved in MeOH (15 mL) and was stirred at 65 °C, for 48 h, until the lower UV-active spot was replaced by a new spot at  $R_f$  0.37 (8% MeOH/CH<sub>2</sub>Cl<sub>2</sub>). The crude material



was purified by column chromatography (0-3% MeOH/CH<sub>2</sub>Cl<sub>2</sub> eluent) to give a yellow solid (485 mg). Analysis by HPLC showed two peaks.

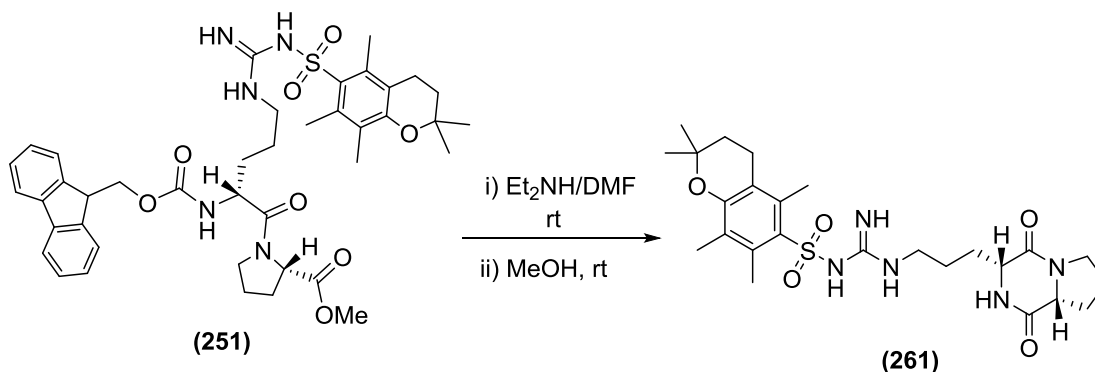
**Method B:** Compound (**249**) (950 mg, 1.22 mmol) was dissolved in anhydrous DMF (2 mL) and treated with Et<sub>2</sub>NH (2.0 mL, 0.19 mol), at room temperature for 30 min, until the starting material was consumed as shown by TLC (8% MeOH/CH<sub>2</sub>Cl<sub>2</sub>). The reaction mixture was evaporated to dryness then co-evaporated with toluene to remove residual DMF to give an oil. The residue was dissolved in MeOH (15 mL) and was stirred at room temperature, for 2 days, until the lower UV-active spot was replaced by a new spot at **R<sub>f</sub>** 0.47 (10% MeOH/CH<sub>2</sub>Cl<sub>2</sub>). The crude material was purified by column chromatography (4-6% MeOH/CH<sub>2</sub>Cl<sub>2</sub> eluent) to give (**259**) as a yellow solid (536 mg, 84%); **RP-HPLC** ((analytical system, gradient 2) **R<sub>t</sub>** = 12.4); [**α**]<sup>19</sup><sub>D</sub> = +9.05 (c 0.53, in MeOH); **Mp** 125-130 °C; **IR** *v*<sub>max</sub> (KBr disc) 3439, 3348 (NH), 2976, 2934 (CH), 1670, 1553 (C=O), 1445 (Ar, NH), 1299, 1246 (CO), 1111 (SO<sub>2</sub>), 744, 666, 614 (CH Ar); **<sup>1</sup>H NMR** (CDCl<sub>3</sub>, 400 MHz) δ 1.28 (6H, s, 2 x Me, Pmc), 1.63-1.66 (2H, m, 2 x H<sub>γ</sub> Arg), 1.76-1.96 (7H, m, CH<sub>2</sub> Pmc, 1 x H<sub>β</sub> Pro, 2 x H<sub>γ</sub> Pro, 2 x H<sub>β</sub> Arg), 2.07 (3H, s, CH<sub>3</sub> from Pmc), 2.27-2.36 (1H, m, 1 x H<sub>β</sub> Pro), 2.51 (6H, m, 2 x, Ar-Me, Pmc), 2.60 (2H, t, *J* 6.7 Hz, CH<sub>2</sub>, Pmc), 3.14-3.26 (2H, m, 2 x H<sub>δ</sub> Arg), 3.42-3.47 (1H, m, 1 x H<sub>δ</sub> Pro), 3.54-3.61 (1H, m, 1 x H<sub>δ</sub> Pro), 3.87-3.91 (1H, m, H<sub>α</sub> Arg), 4.09-4.13 (1H, m, H<sub>α</sub> Pro), 6.38 (3H, br, 3 x NH-Arg), 7.76 (1 H, br, NH-Arg); **<sup>13</sup>C NMR** (CDCl<sub>3</sub>, 101 MHz) δ 12.05, 17.37, 18.42 CH<sub>3</sub> (Pmc, Me), 21.33 (2 x CH<sub>2</sub> Pmc), 21.99 (CH<sub>2</sub>γ Pro, CH<sub>2</sub>γ Arg), 25.31, 26.72 CH<sub>3</sub> (Pmc, Ar-Me) 28.92 (CH<sub>2</sub>β Pro, CH<sub>2</sub>β Arg), 31.05 (CH<sub>2</sub>δ Arg), 40.11 (CH<sub>2</sub>δ Pro), 45.51 (CH<sub>α</sub> Arg), 56.92 (CH<sub>α</sub> Pro), 58.04 (OCH<sub>3</sub>), 73.67 (C quaternary Pmc), 118.02, 124.09, 133.01, 134.73, 135.31, 153.66 (C quaternary), 156.30, 166.19, 169.58 (2 x C=O, C=N); [found (ES<sup>+</sup>) 542.2425 [M+Na]<sup>+</sup>, C<sub>25</sub>H<sub>37</sub>N<sub>5</sub>O<sub>5</sub>Na requires 542.2413].

### Cyclo (D-Arg(Pmc)-L-Pro) (260)



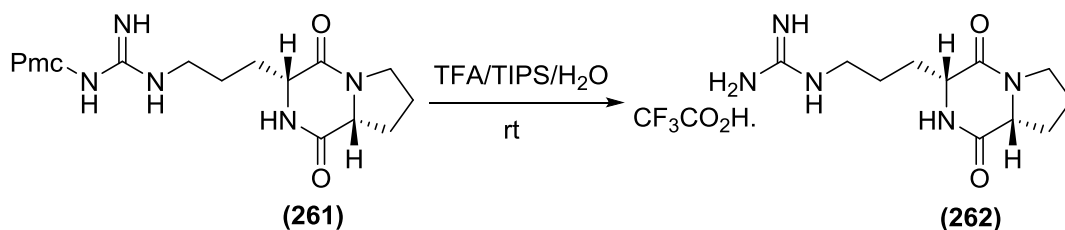
Compound (**250**) (843 mg, 1.09 mmol) was dissolved in anhydrous DMF (2 mL) and treated with Et<sub>2</sub>NH (2.0 mL, 0.19 mol), at room temperature for 30 min, until the starting material was consumed as shown by TLC (8% MeOH/CH<sub>2</sub>Cl<sub>2</sub>). The reaction mixture was evaporated to dryness then co-evaporated with toluene to remove residual DMF to give an oil. The residue was dissolved in MeOH (15 mL) and was stirred at room temperature, for 2 days, until the lower UV-active spot was replaced by a new spot at *R<sub>f</sub>* 0.44 (10% MeOH/CH<sub>2</sub>Cl<sub>2</sub>). The crude material was purified by column chromatography (5-9% MeOH/CH<sub>2</sub>Cl<sub>2</sub> eluent) to give (**260**) as a yellow solid (494 mg, 87%); **RP-HPLC** (analytical system, gradient 2) *R<sub>t</sub>* = 12.4; [ $\alpha$ ]<sup>19</sup><sub>D</sub> = -9.42 (c 0.45, in MeOH); **Mp** 123-128 °C; **IR**  $\nu_{\text{max}}$  (KBr disc) 3439, 3343 (NH), 2973, 2936 (CH), 1659, 1571 (C=O), 1447 (Ar, NH), 1299, 1245 (CO), 1110 (SO<sub>2</sub>), 747, 666, 614 (CH Ar); **<sup>1</sup>H NMR** (CDCl<sub>3</sub>, 270 MHz)  $\delta$  1.28 (6H, s, 2 x Me, Pmc), 1.63-1.68 (2H, m, 2 x H $\gamma$  Arg), 1.76-1.99 (7H, CH<sub>2</sub> Pmc, 1 x H $\beta$  Pro, 2 x H $\beta$  Arg, 2 x H $\gamma$  Pro), 2.15 (3H, s, CH<sub>3</sub> from Pmc), 2.29-2.32 (1H, m, 1 x H $\beta$  Pro), 2.51 (6H, m, 2 x Ar-Me, Pmc), 2.60 (2H, t, *J* 6.7 Hz, CH<sub>2</sub>, Pmc), 3.14-3.26 (2H, m, 2 x H $\delta$ -Arg), 3.43-3.47 (1H, m, 1 x H $\delta$  Pro), 3.54-3.61 (1H, m, 1 x H $\delta$  Pro), 3.88-3.92 (1H, m, H $\alpha$  Arg), 4.09-4.13 (1H, m, H $\alpha$  Pro), 6.35 (3H, br, 3 x NH-Arg), 7.71 (1 H, br, NH-Arg); **<sup>13</sup>C NMR** (CDCl<sub>3</sub>, 101 MHz)  $\delta$  12.06, 17.37, 18.42 CH<sub>3</sub> (Pmc, Me), 21.35 (2 x CH<sub>2</sub> Pmc), 21.99 (CH<sub>2</sub> $\gamma$  Pro, CH<sub>2</sub> $\gamma$  Arg), 25.31, 26.71 CH<sub>3</sub> (Pmc, Ar-Me) 28.89 (CH<sub>2</sub> $\beta$  Pro, CH<sub>2</sub> $\beta$  Arg), 31.06 (CH<sub>2</sub> $\delta$  Arg), 40.15 (CH<sub>2</sub> $\delta$  Pro), 45.51 (CH $\alpha$  Arg), 56.95 (CH $\alpha$  Pro), 58.03 (OCH<sub>3</sub>), 73.67 (C quaternary Pmc), 118.03, 124.10, 133.00, 134.72, 135.28, 153.66 (C quaternary), 156.32, 166.21, 169.52 (2 x C=O, C=N); [found (ES<sup>+</sup>) 542.2336 [M+Na]<sup>+</sup>, C<sub>25</sub>H<sub>37</sub>N<sub>5</sub>O<sub>5</sub>Na requires 542.2408].

### Cyclo (D-Arg(Pmc)-D-Pro) (261)



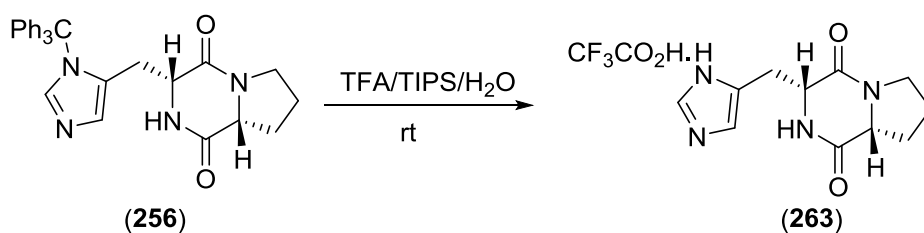
Compound (**251**) (320 mg, 0.413 mmol) was dissolved in anhydrous DMF (2 mL) and treated with Et<sub>2</sub>NH (2.0 mL, 0.19 mol), at room temperature for 30 min, until the starting material was consumed as shown by TLC (8% MeOH/CH<sub>2</sub>Cl<sub>2</sub>). The reaction mixture was evaporated to dryness then co-evaporated with toluene to remove residual DMF to give an oil. The residue was dissolved in MeOH (15mL) and was stirred at room temperature, for 2 days, until the lower UV-active spot was replaced by a new spot at **R<sub>f</sub>** 0.42 (10% MeOH/CH<sub>2</sub>Cl<sub>2</sub>). The crude material was purified by column chromatography (0-6% MeOH/CH<sub>2</sub>Cl<sub>2</sub> eluent) to give (**261**) as a yellow oil (165 mg, 77%); **RP-HPLC** (analytical system, gradient 2) **R<sub>t</sub>** = 12.5 min); [**α**]<sup>19</sup><sub>D</sub> = +2.91 (c 3.81, in MeOH); **IR**  $\nu_{\text{max}}$  (thin film) 3442, 3337 (NH), 2976, 2936 (CH), 1670, 1579 (C=O), 1442 (Ar, NH), 1298, 1244 (CO), 1110 (SO<sub>2</sub>), 754, 665 (CH Ar) **<sup>1</sup>H NMR** (CDCl<sub>3</sub>, 400 MHz)  $\delta$  1.27 (6H, s, 2 x Me, Pmc), 1.61-1.74 (2H, m, 2 x H $\gamma$  Arg), 1.76 (2H, t, *J* 6.7 Hz, CH<sub>2</sub>, Pmc), 1.82-2.04 (5H, m, 2 x H $\gamma$  Pro, 2 x H $\beta$  Arg, 1 x H $\beta$  Pro), 2.06 (3H, s, CH<sub>3</sub> from Pmc), 2.28-2.32 (1H, m, 1 x H $\beta$  Pro), 2.51 (6H, m, 2 x CH<sub>3</sub>, Ar-Me Pmc), 2.58 (2H, t, *J* 6.6 Hz, CH<sub>2</sub>, Pmc), 3.16-3.17 (2H, m, 2 x H $\delta$  Arg), 3.43-3.55 (2H, m, 2 x H $\delta$  Pro), 4.04-4.08 (2H, m, H $\alpha$  Arg, H $\alpha$  Pro), 6.32 (2.6H, br, 2.6 x NH Arg), 7.45 (0.85 H, br, 0.85 x NH Arg); **<sup>13</sup>C NMR** (CDCl<sub>3</sub>, 101 MHz)  $\delta$  12.05, 17.39, 18.43 CH<sub>3</sub> (Pmc, Me), 21.34 (2 x CH<sub>2</sub> Pmc), 22.01 (CH<sub>2</sub> $\gamma$  Pro, CH<sub>2</sub> $\gamma$  Arg), 26.70, 26.73 (Ar-Me, Pmc) 28.20 (CH<sub>2</sub> $\beta$  Pro, CH<sub>2</sub> $\beta$  Arg), 32.74 (CH<sub>2</sub> $\delta$  Arg), 40.70 (CH<sub>2</sub> $\delta$  Pro), 45.23 (CH $\alpha$  Arg), 54.86 (CH $\alpha$  Pro), 59.02 (OCH<sub>3</sub>), 73.60 (C quaternary Pmc), 117.95, 123.96 133.25, 134.75, 135.33 153.52 (C quaternary), 156.40, 165.69, 170.73 (2 x C=O, C=N); [found (ESI<sup>+</sup>) 520.2605 [M+H]<sup>+</sup>, C<sub>25</sub>H<sub>38</sub>N<sub>5</sub>O<sub>5</sub> requires 520.2593].

### Cyclo(D-Arg-D-Pro) (262)



Compound **(261)** (37 mg, 0.071 mmol) was treated with a solution of TFA/TIPS/H<sub>2</sub>O (95:2.5:2.5, v/v/v) (1 mL) and stirred at room temperature for 3 h. The reaction mixture was concentrated under vacuum and the residue dissolved in H<sub>2</sub>O (10 mL) and extracted with CH<sub>2</sub>Cl<sub>2</sub> (3 x 10 mL). The organic layer was dried, filtered and the solvent evaporated to give **(262)** as an oil (33 mg, quantitative yield); <sup>1</sup>H NMR (CD<sub>3</sub>OD, 270 MHz) δ 1.66-1.68 (2H, m, 2 x H<sub>γ</sub> Arg), 1.91-1.98 (5H, 1 x H<sub>β</sub> Pro, 2 x H<sub>γ</sub> Pro, 2 x H<sub>β</sub> Arg), 2.30-2.33 (1H, m, 1 x H<sub>β</sub> Pro), 3.18-3.27 (2H, m, 2 x H<sub>δ</sub> Arg), 3.50-3.59 (2H, m, 2 x H<sub>δ</sub> Pro), 4.22 (2H, br, H<sub>α</sub> Arg, H<sub>α</sub> Pro); [found (ES<sup>+</sup>) 254.1592 [M+H]<sup>+</sup>, C<sub>11</sub>H<sub>20</sub>N<sub>5</sub>O<sub>2</sub> requires 254.1617].

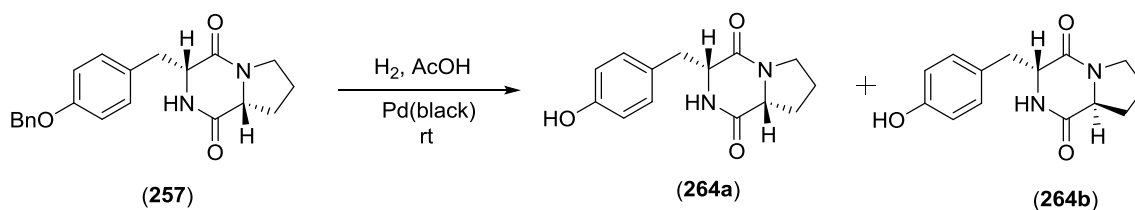
### Cyclo (D-His-D-Pro) (263)



Compound **(256)** (41 mg, 0.086 mmol) was treated with a solution of TFA/TIPS/H<sub>2</sub>O (95:2.5:2.5, v/v/v) (1 mL) and stirred at room temperature for 1 h; additional TIPS (252 μL, 1.22 x 10<sup>-3</sup> mmol) was then added until the reaction mixture was colourless. The reaction mixture was evaporated to dryness and the residue dissolved in H<sub>2</sub>O (10 mL) and extracted with CH<sub>2</sub>Cl<sub>2</sub> (3 x 10 mL). The organic layer was dried, filtered and the solvent was evaporated to give **(263)** as an oil (34 mg, quantitative yield); **RP-HPLC** (analytical system, gradient 1) R<sub>t</sub> = 2.6 min; **IR** ν<sub>max</sub> (thin film) 3306 (NH, OH), 2892 (CH), 1674 (C=O), 1437 (NH), 1198, 1136 (CO); <sup>1</sup>H NMR (CDCl<sub>3</sub>, 270 MHz) δ 1.88-2.00 (3H, m, 2 x H<sub>γ</sub> Pro, 1 x H<sub>β</sub> Pro), 2.03-2.07

(1H, m, H $\beta$  Pro), 2.15-2.28 (1H, m, 1 x H $\beta$  His), 3.30-3.47 (3H, m, 1 x H $\beta$  His, 2 x H $\delta$ -Pro), 4.23 (1H, br, H $\alpha$  His), 4.56 (1H, br, H $\alpha$  Pro), 7.42 (1H, br, NH), 7.90 (1H, br, 1H imidazole ring), 8.83 (1H, br, NH);  $^{13}\text{C}$  NMR (CDCl<sub>3</sub>, 101 MHz)  $\delta$  22.19 (CH<sub>2</sub> $\gamma$  Pro), 24.80 (CH<sub>2</sub> $\beta$  Pro), 28.08 (CH<sub>2</sub> $\beta$  His), 45.07 (CH<sub>2</sub> $\delta$  Pro), 54.58 (CH $\alpha$  His), 58.9 (CH $\alpha$  Pro), 118.10 (CH=C), 129.35 (C quaternary), 133.33 (CH=C), 165.04, 169.96 (C=O) 176.17 (CO<sub>2</sub>H), 205.59 (CF<sub>3</sub>); [found (ES<sup>+</sup>) 235.1162 [M+H]<sup>+</sup>, C<sub>11</sub>H<sub>20</sub>N<sub>5</sub>O<sub>2</sub> requires 235.1190].

### Cyclo(D-Tyr-D-Pro) (264)



Compound (**257**) (20 mg, 0.06 mmol) was dissolved in AcOH (1 mL) and then was treated with Pd (black) (1.5 mg) and the reaction mixture was stirred at room temperature under a H<sub>2</sub> atmosphere. The reaction was monitored by TLC. After running the reaction for 4 h 30, the starting material was consumed and a new spot at **R<sub>f</sub>** 0.55 (10% MeOH/CH<sub>2</sub>Cl<sub>2</sub>) appeared. The reaction mixture was filtered through Celite, diluted in EtOH (15 mL) then co-evaporated with Et<sub>2</sub>O to give a mixture of diastereoisomers, (**264a**) and (**264b**) as an oil (10.3 mg, 66%); **IR**  $\nu_{\text{max}}$  (thin film) 3260 (NH), 1666 (C=O), 1438 (NH), 1239 (CO);  $^1\text{H}$  NMR (CD<sub>3</sub>OD, 400 MHz)  $\delta$  1.80-2.02 (2H, m, 2 x H $\gamma$  Pro), 2.04-2.09 (2H, m, 2 x H $\beta$  Pro), 3.03-3.12 (2H, m, 2 x H $\beta$  Tyr), 3.35-3.56 (2 H, m, 2 x H $\delta$  Pro), 3.91-3.98 (0.2 H, m 0.2 x H $\alpha$  Pro), 4.04-4.07 (0.8 H, m, 0.8 x H $\alpha$  Pro), 4.36 (0.8 H, m, 0.8 x H $\alpha$  Tyr), 4.43 (0.2 H, m, 0.2 x H $\alpha$  Tyr), 6.69-6.71 (2H, m, ArH), 7.03-7.05 (2H, m, ArH).

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